Ms. Leslie Furr
Associate Scientific Liaison
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[redacted]

Craponne October 16th, 2020

RE: General Chapter <1085.1> Use of Recombinant Reagents in the Bacterial Endotoxins Test—Photometric and Fluorometric Methods Using Recombinantly Derived Reagents

Dear Ms. Furr:

We are writing in response to the draft Guidance posted 01-Sep-2020 for a new General Chapter <1085.1>, *Use of Recombinant Reagents in Bacterial Endotoxins Test.*

Our company has been producing the recombinant Factor C (rFC) biosensor protein for several years as an equivalent alternative to the biologically sourced *Limulus* amebocyte lysate (LAL) assay. We have validated that rFC is equivalent and comparable to LAL when tested in a suitable water, raw material, or drug matrix. We have found that rFC is equivalent to LAL in detecting bacterial endotoxins in injectable pharmaceuticals and is advantageous for manufacturers to use to ensure the safety of injectable products.

We were in favor of revising compendial chapter <85>, in a manner that would follow the EMQD in creating a stand-alone chapter to direct the use of rFC (with the ultimate goal of harmonizing both with Ph.Eur. 2.6.32 and <85>), instead USP created a guidance chapter that creates many more barriers to adopting rFC relative to the status quo. In some cases the proposed changes are in excess of current requirements for LAL manufacture, for example:

- USP draft <1085.1> requests control of "the type and control of cell lines used" which exceeds the characterization of LAL. A corollary from a natural product would be the determination of the horseshoe crab genome sequences which generate the cascade proteins. A wild-type genome is known to contain variability and mutations (the basis of evolution), organism to organism, over time. LAL manufacturers do not confirm the appropriate genetic sequences for horseshoe crabs caught from the sea and bled in the factory. In fact, this is a good argument in favor of rFC in that these sequences have been cloned and controlled over time in a scientific manner.
- Similarly, the USP draft <1085.1> statement: "identification and impact of any post-translational modification of the recombinant zymogen proteases" would

require the routine check of the Factor C protein glycosylation pattern while leaving completely unknown the routine configuration of the multiple native LAL proteins as harvested and formulated into various LALs. Neither is there knowledge around the effect of LAL chloroform extraction on glycosylation of all the LAL proteins. No one knows the detailed molecular contents of LAL, another argument that favors the use of rFC.

Unfortunately, this USP action adds unnecessary delay to incorporating rFC in a USP General Method. We hope that USP will further engage all stakeholders and help to create a timely path forward toward approval of a compendial rFC method and improve patient safety. We would prefer that you seek to directly enact a General Chapter similar to the European Pharmacopeia Ph.Eur. 2.6.32, either by amending <85> or creating a new sub-1000 chapter, (i.e. <86>). With regard to informational chapter <1085.1>, we feel obliged to point out significant misleading issues with the proposed chapter that must be resolved before it reaches its final form.

Importantly, the proposed comparability guidance goes beyond FDA guidelines and published evidence for comparability and equivalence of the assays. Indeed, the additional work USP is now proposing emerges not from weaknesses in rFC, but from the variability of LAL due to its biological origins, largely uncharacterized contents and multiple manufacturing methods (e.g. chloroform extractions vs. zwittergent addition, etc.), and because LAL is subject to a range of artifacts, not least the enhancement of positive signals by the presence of glucans (synergistic effects of glucans on endotoxin). Indeed, one could conclude from your document that it is LAL that needs to be proven against rFC's high sensitivity, reproducibility simplicity, and elimination of false positives. In short, you are asking for rFC to achieve comparability with a more complex, less reproducible assay that is subject to elusive false positives. This unique situation needs to be examined and rectified in terms of making forward progress that is not biased toward the LAL status quo.

We request that USP adjust their efforts by taking into account the following comments:

- Remove from the proposed chapter <1085.1> occurrences of additional validation proof beyond what is already stated in 2012 FDA Guideline and <1225>, specifically the proposed comparability guidance. The addition of references to <1223> which is typically used to validate microbiological enumeration and rapid methods completely confuses the validation issue.
- Remove references to performing validation using non-purified waters, including deionized water, which are not controlled for bacterial counts, total organic carbon (TOC) or glucans and other organic background that is known to interfere with LAL methods. These source waters are not currently tested for endotoxin.
- Remove from the proposed chapter <1085.1> the PTC on supplier quality, including Table 1, as that is out of scope of public health standards.
- Establish in short order a sub <1000> standalone chapter similar to Ph.Eur. 2.6.32, that can ultimately be merged into <85>, and which, importantly recognizes the scientific validity and comparability of rFC as does the EMQD.

- Remove references of the comparison of LAL with rabbit pyrogen testing as inclusion of BET in the pharmacopoeia was a very different situation in moving from a pyrogen test to a endotoxin test. The inclusion of rFC in the BET chapter or its own compendial chapter is based on the comparison or LAL to rFC for endotoxin detection assays (not pyrogen assays).
- Acknowledge that FDA has approved drug products based upon alternative validation performed as per USP <1225>.

We look forward to engaging with you further on this issue and will reach out to discuss how these matters may best be moved forward in discussions with you.

Lonza

16 Oct. 2020

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Dear Dr. Furr,

Lonza is pleased to be provided an opportunity to comment on this critical General Informational Chapter. Please see the attached comments to the proposed Chapter: <1085.1> Use of Recombinant Reagents in Bacterial Endotoxins Test.

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[redacted],
[redacted]
[redacted]
Lonza Walkersville, Inc.

/comments attachment

Comments to USP <1085.1>

Section and paragraph	Passage for comment	Comment on passage
Briefing	…requires demonstration of comparability based on criteria recommended in this chapter proposal and other USP chapters, principally Validation of Alternative Microbiological Methods ⟨1223⟩, Validation of Compendial Procedures ⟨1225⟩, and Guidelines on the Endotoxins Test⟨1085⟩ as noted.	Validation according to <1223> is not appropriate for an enzyme-based assay. <1225> and <1085> are appropriate.
Briefing	This proposed chapter also provides suggestions for the evaluation of a recombinant reagent supplier.	Supplier qualification should not be part of pharmacopeial method description. We suggest it be removed or substitute by reference to appropriate guideline.
Briefing	The recombinant reagents described in this chapter ~~are not sourced from lysates of amebocytes, but rather~~ use one or more cloned zymogen proteases that comprise all or part of the natural lysate reaction cascade (Figure 1).	The stricken language creates a negative tone, so we suggest it be deleted. Is this chapter meant for release testing only? We would recommend to at least mention if this whole chapter is intended for release testing only and if yes, the title or briefing must be more clear on this. The introduction is missing that the immunological mechanisms are conserved among HSC species . However, lysate preparation varies among the manufacturers indicating that results are valid in the range of 50-200%. Throughout the document we would suggest to remove the phrase "naturally lysate" as this is non-existing. The amebocyte blood is naturally sourced while the preparation of the lysate is a manufacturing process that differs between manufacturers. It is well known that this is leading to differences in reactivity patterns across all compendial methods.
Introduction	These characteris ics give the horseshoe crab immune system high sensi ivity to an extremely broad spectrum of Gram-negative bacteria with which these species have coevolved.	The sensitivity is to a broad spectrum of (marine) endotoxins. We recommend to add for scientific correctness and for providing a rationale for the requirement to test autochthonous endotoxin in manufacturing facilities. (this is different from marine environment). This should not be limited to recombinant reagents.
Background (compendial methods)	Explain factor G pathway (currently only mentioned in background recombinant methods) along with the graph shown	The graph shown mentions the factor G path. We would recommend to describe the factor G path here instead of mentioning it in "background – recombinant reagents" for the first time. We would further suggest to move the below sentence from "background recombinant methods" to "background compendial methods": "When a sample contains sufficient β-D-glucan, the presence of the Factor G pathway in natural lysate can result in an overestimation of endotoxins activity (*5*–*6*)."
Background (recombinant methods)	Recombinant bacterial endotoxins test (BET) reagents proposed as alternatives to naturally sourced Limulus amebocyte lysate (LAL).	This section is misleading, and not in line with <85>. We suggest to either include TAL or keep it broad to include all lysates derived from natural sources (to include all horseshoe crab species).
Background (recombinant methods)	These tests require the use of a qualified and calibrated fluorometer for reading the signal.	This is misleading, as the reference to qualified and calibrated instrumentation applies to all photometric **AND** fluorometric assays. We suggest to change to "*Photometric and recombinant reagents require the use of instrumentation qualified, calibrated and properly maintained according to USP<85> and <1058>*". It is further required to qualify any reagent on a reader (IQ/OQ/PQ) therefore this is not limited to

		rFC reagents or a fluorochrome read-out. At least, it applies to both rFC and rFC reagents. Additionally, this route seems to require a data integrity statement.
Background (recombinant methods)	The test methodology for using rCR is the same as the kinetic chromogenic method and is performed as described in ⟨85⟩, Photometric Quantitative Techniques, Chromogenic Technique.	We find it very problematic to describe rCR at this level of detail based on suppliers marketing materials. There is no independent user experience or peer-reviewed data on these products We recommend to limit this chapter to rFC where there is sufficient, independent and real world data available in the public domain. Alternatively, since rCR products are not yet available to the US market we would like to get access to the data from public resources that are supporting these claims.
Background (recombinant methods)	There are benefits to the adoption of recombinantly derived reagents: ▪ The elimination of the use of animals as a reagent source ▪ The absence of the glucan pathway that can result in a non-endotoxin-specific enhancement of the lysate test result ▪ The potential for a more consistent and stable supply of reagent not subject to market and environmental pressures	Benefits: this section is in wrong position, also the wording indicates that applies to rCR only. We would suggest to place it at the beginning of this section and to replace "recombinantly derived" by rFC and rCR.
Alternative methods	The alternative method or procedure must be fully validated (see Validation of Compendial Procedures ⟨1225⟩) and must produce comparable results to the compendial method or procedure within allowable limits established on a case-by-case basis."	Essentially there is no marked difference in procedures between recombinant reagents and compendial reagents. However, each of the compendial reagents and recombinant reagents may display different reactivity patterns with any given product especially when it comes to interference patterns or unspecific reactivity, e.g. to glucans. Therefore a product-specific validation approach is highly recommended and is already performed in real life with the compendial reagents from one or more manufacturers. We do not see the necessity for an alternative method validation for recombinant reagents. By their very nature recombinant reagents may react more specifically to Endotoxin than compendial reagents which makes it difficult to identify a "true" value. With this in mind we believe that the concurrent chapter for alternative method validation might not be necessary.
Alternative methods	Although the instructions for performing the recombinant methods are essentially identical to the endpoint- and kinetic- chromogenic assays described in ⟨85⟩, the reagents are *not* identical to reagents prepared from horseshoe crab hemolymph. Therefore, endotoxins tests using recombinant reagents are considered to be *alternative methods*.	It's well worth pointing out that the lysates produced by different vendors and for different methods are also far from identical and have been demonstrated to give differing results. In fact no two sets of lysate are identical, especially if we consider turbidimetric and chromogenic lysates. Therefore the argument that recombinant technology that uses exactly the same binding protein to initiate the response should be an alternative method has never been a convincing argument.
Validation of Alternative Methods	Prior to validation of an endotoxins test using recombinant reagents, a user requirement specification (URS) should be produced per Validation of Alternative Microbiological Methods ⟨1223⟩.	Alternative method validation according to <1223> is not justified. Endotoxin assays belong to "biological test and assays" and not "alternative microbiological tests" (with reference to USP table of contents). Most of what is requested for <1223> is done by the suppliers and should not be requested from users. We suggest that the reference to <1223> for the entire document be deleted.
Validation of Alternative Methods	Reagent feasibility studies, instrument qualifications, and other activities performed to support the choice of an alternative reagent prior to the execution of the	URS, reagent qualification, instrument qualification etc. is a general requirement for choosing a test method (e.g. for product safety testing) and not limited to recombinant reagents. Further, these

	final studies should be documented as part of the overall validation effort	requirements are more specifically handled in USP chapters other than <1223>. We suggest this section be stricken.
Validation of Alternative Methods	3. Comparability: Given the complexity of the lysate prepared from disrupted amebocyte granules, comparability of the recombinant reagents to naturally sourced lysate using endotoxins from autochthonous manufacturing sources is of particular importance. Comparability of recombinant reagents should therefore demonstrate equivalency of results per ⟨1223⟩. (For further information, see Comparability.) Current literature suggests that the following may affect the sensitivity and/or specificity of the recombinant reagents and should be considered when choosing reagent suppliers (see below) and preparing comparability protocols	There is no such thing than natural sourced lysate but a lysate prepared from a natural resource which is variable in itself. This is a huge difference since the different preparation protocols from suppliers may lead to different reactivity towards autochthonous bacteria. This point is therefore not limited to recombinant reagents. Additionally, reference to <1223> is not justified because this is not a microbiological assay. . To our perspective none of the points 1-7 apply as they are part of the manufacturers deign qualification (DQ) and should not be part of demonstrated equivalency. Moreover most literature chosen refer to discovery data in artificial systems that were not verified by relevant studies.
Validation of alternative methods	(1) Choice of species of the horseshoe crab as the source of the recombinant reagents (11)	The current compendial document allows the use of TAL and LAL interchangeably, despite demonstrable differences in the reagents and even their mode of operation e.g. cleavage of the coagulogen in two places in the case of *Tachypleus* lysate gel clot reagents. It is difficult therefore to understand why the choice of the species of HSC should be need to be justified as part of a validation for alternative methods. If we also look at other types of alternative method validation, PCR for example, potential users are not asked to justify the source or even the type e.g. hot start, cold start etc. of Taq polymerase. We suggest this section be stricken.
Validation of alternative methods	(2) Optimal number of cascade zymogen proteases in the recombinant reagent formulation (*13*–*14*)	Given that the primary purpose of the enzyme cascade is to amplify the initial signal to a detectable level, asking potential users to justify the 'optimal' number of zymogen proteases is both unnecessary, scientifically unsound and unduly onerous. In fact there is no 'optimal' number provided the signal generated by the reagents is sufficient to give an adequate measurable range and suitable Limit Of Detection (LOD). It is the task for each vendor of recombinant reagents to formulate the final product and to state its documented LOD. The user already has to confirm the manufacturer's LOD as part of the validation procedure. We suggest this section be stricken.
Validation of alternative methods	(3) The type and control of cell lines used as the expression system (*10*–*11*)	Once again the requirements on the potential user of recombinant reagents goes way beyond what would normally be required for the validation of an alternative method. In assessing comparability, would the authors like to state what relevance this requirement has in terms of the selection of a set of reagents? Much of this information would be proprietary and has little or no bearing on the ability of the reagents to detect endotoxin or their performance in an assay. This information would be supplied to regulatory agencies in the form of a Master File, as Lonza has already done many years ago. We suggest this section be stricken.
Validation of alternative methods	(4) Identification and impact of any post-translational modification of the recombinant zymogen proteases (*10*–*11*, *15*–*16*)	This seems to be a request for proprietary information that is only relevant to a drug product that will be administered to humans or animals. If requested by a regulator, this information would be provided to that regulator in a Master File, not to the customer. We suggest this section be stricken.
Validation of alternative methods	(5) The final, proprietary reagent formulation that assures consistent functionality and stability of the chosen recombinant components selected by the reagent manufacturer (*10*)	Yet again the onus is placed on potential users of recombinant product to ask for proprietary information that is not required for the many different forms of HSC lysate that the user can switch between at will, provided they have validated that

		product for the method. If required by a regulator, this information would be submitted to that regulator in a Master File. We suggest this section be stricken.
Validation of alternative methods	(6) In addition, unlike with conventional lysate reagents described in ⟨85⟩, the manufacture, use, and distribution of recombinant reagents is *not* regulated by any regional authority.	Many of the required tests for product release such as reagents and accessories for the sterility test are not regulated by any regional authorities. We suggest that this be stricken.
Validation of alternative methods	(7) Therefore, it is incumbent on the user of these reagents to assure that the manufacture, distribution, storage, stability, and use of the selected recombinant reagent provides sufficient controls to result in *consistently comparable results* to the naturally sourced LAL reagents.	Users are not required to undertake extensive validation of the manufacture, distribution, storage, stability, and use of these reagents, so why is this unduly onerous requirement introduced for recombinant reagent? We suggest that this be stricken.
Validation of alternative methods	It is suggested that users of recombinant reagents conduct preliminary studies to determine if a change to a recombinant method is feasible. Data from preliminary studies may need to be submitted to the regional regulatory authority as part of the method validation package.	The requirements for this statement are unclear. Chapter <85> specifies the requirements for product validation. There is no description of what these 'preliminary studies' should comprise. This vague and unhelpful comment will only create uncertainty as to how to proceed.
Preparatory Testing and General Notes	Comment on section	This section should be moved before "alternative method validation"
Preparatory Testing and General Notes	Apparatus: Reference ⟨85⟩. For fluorometric tests, qualify instruments per Analytical Instrument Qualification ⟨1058⟩ and calibrate the instrument according to the manufacturer's instructions.	This is not limited to fluorometric instruments but to all instrumentation. Chapter <85> refers to manufacturer instructions, no reason to limit these requirements to recombinant reagents. We recommend deleting "for Fluorometric tests"
Preparatory Testing and General Notes	Reagents and test solutions: Reference ⟨85⟩ except for recombinant reagents. For those, follow the manufacturer's instructions for storage, reconstitution, and use.	<85> refers to manufacturer instructions, no reasons to limit to recombinant reagents. We would recommend to delete the exception to recombinant reagents.
Preparatory Testing and General Notes	Preparatory testing (assurance of criteria for the calibration curve): Reference ⟨85⟩*, Photometric Quantitative Techniques, Chromogenic Technique*. • rFC assay: reference the endpoint-chromogenic assay in ⟨85⟩. • rCR assay: reference the kinetic-chromogenic assay in ⟨85⟩.	Chapter <85> doesn't make any differentiation between kinetic or endpoint, or chromogenic and turbidimetric. It is the same text for all, titled PHOTOMETRIC QUANTITATIVE TECHNIQUES - Preparatory Testing - assurance of criteria for the standard curve. We would recommend to adjust the title and delete the bullet points.
Test for interfering factors	As with the natural lysate reagents, the user of recombinant reagents is required to assure that the material under test neither enhances nor inhibits the detection or quantification of endotoxin activity with the chosen reagent.	There is no natural lysate. Recommend to delete "natural". It is a well known and documented fact that interference patterns can vary greatly for different lysates and/or lysate from different vendors for the same product. Why then is the weak '*formulation components of recombinant reagents are not identical to the naturally sourced lysate reagents'* argument restated here. We have no argument with the need to determine interference patterns with rFC methods and to take appropriate steps to overcome that interference but see no difference between lysate based and rFC based methods. We believe the 2nd paragraph is therefore unnecessary and should be struck.
Test for interfering factors	Because the formulation components of recombinant reagents are not identical to the naturally sourced lysate reagents there is no reason to expect that the interference patterns using the lysate and recombinant methods will be the same (4,17–18).	There is no naturally sourced lysate. We recommend that the term "naturally sourced" be stricken. Again, this would also be true for chromogenic vs turbidimetric, or lysates from different manufacturers. A product might not show the same interference profile when you change from one LAL

		to the other, which is why the end-user must revalidate when changing suppliers.
Test for interfering factors	Depending on the product, the non-interfering concentration/dilution may be different between the recombinant and natural lysate methods. In some cases, one or the other of the test methods might require significant changes to the sample preparation (19).	There is no natural lysate method. We recommend to delete "natural". The non-interfering dilutions differs between lysates from different manufacturers and between compendial methods using lysate will differ therefore it is wrong to phrase it as a difference between recombinant reagents and lysate reagents. We recommend: "Depending on the product, the non-interfering concentration/dilution may vary within the range of compendial lysate methods and between lysate methods and recombinant methods. In some cases, one or the other of the test methods might require significant changes to the sample preparation"
Test for interfering factors	The test procedure for the *Test for Interfering Factors* using rFC and rCR reagents is the same as ⟨85⟩*, Photometric Quantitative Techniques, Preparatory Testing, Test for Interfering Factors* for the endpoint-chromogenic and kinetic-chromogenic techniques, respectively.	This is one section for all photometric methods. There is no reason to construct a difference not described in this chapter. We would recommend to close the sentence after the title of the chapter.
Comparability	Any scientifically justified protocol designed specifically to compare *assayable levels* of autochthonous endotoxins from manufacturing sources to user-defined and predetermined acceptance criteria may be utilized.	Even autochthonous endotoxins from manufacturing sources may differ based on the environmental conditions. Therefore, we find it problematic to indicate that there are absolute values. Moreover, authors neglect standard requirements outlined in general for microbiological methods which is to assess 3-5 dominant house organisms as most representative autochthonous endotoxins.
Comparability	Historically, prior to the acceptance of the LAL method as comparable to the rabbit pyrogen test it replaced, comprehensive studies were performed to assure that the LAL method could provide equivalent (or better) product quality decisions (20).	We are not aware that the LAL assay completely replaced the RPT therefore we would recommend to limit to certain applications where Endotoxin is the only object.
	Currently, although data are available on suitability (inhibition/enhancement) testing using recombinant reagents, comprehensive data demonstrating comparability of recombinant methods to LAL lysates in compendial articles containing assayable levels of endotoxins activity from autochthonous endotoxins are not available or have not been published in the public domain. Therefore, until such data become available it is up to each stakeholder who wishes to qualify a recombinant BET to undertake appropriate comparability trials.	Please consider the following peer-reviewed journal articles: Bolden J, Smith K. Application of recombinant factor C reagent for the detection of bacterial endotoxins in pharmaceutical products. PDA J Pharm Sci Technol 2017;71:405–12. Abate W, Sattar AA, Liu J, Conway ME, Jackson SK. Evaluation of recombinant factor C assay for the detection of divergent lipopolysaccharide structural species and comparison with Limulus amebocyte lysate-based assays and a human monocyte activity assay. J Med Microbiol 2017;66:888–97. Kikuchi Y, Haishima Y, Fukui C, Murai T, Nakagawa Y, Ebisawa A, et al. Collaborative study on the bacterial endotoxins test using recombinant factor Cbased procedure for detection of lipopolysaccharides. PMDRS 2017;48:252–60. Marius M, Vacher F, Bonnevay T. Comparison of LAL and recombinant factor C endotoxin testing assays in human vaccines with complex matrices. PDA J Pharm Sci Technol 2020. pdajpst.2019.010389. Piehler M, Roeder R, Blessing S, Reich J. Comparison of LAL and rFC assays-participation in a proficiency test program between 2014 and 2019. Microorganisms 2020:8.

		Bolden JS, Warburton RE, Phelan R, Murphy M, Smith KR, De Felippis MR, et al. Endotoxin recovery using limulus amebocyte lysate (LAL) assay. Biologicals 2016;44:434–40.
Comparability	In addition to using samples "spiked" with the current USP Endotoxin RS (RSE) or commercially prepared control standard endotoxins (CSE) during *the Test for Interfering Factors*, it is recommended to also compare test results using products that contain known levels of measurable endotoxins activity from a source that reasonably could be expected to contaminate the product. In other words, comparability cannot be demonstrated by using only test articles that do not contain levels of assayable endotoxins. CSEs or RSEs may be used in addition to autochthonous endotoxins if required by a regional regulatory authority. If available, samples from actual production runs that contain assayable levels of endotoxins could be used.	Once again the potential user of recombinant reagents is faced with a significant amount of additional work. Worse, there is no definition of what might be accepted by a regulatory authority, as the text does not specify how many products types should be tested, nor how many samples are considered a reasonable number. Furthermore, in a well-run facility operating under GMP, the number of potential samples that would have assayable endotoxin, are likely to be very low or at the bottom end of the detectable range, making statistical accuracy for measurement of these samples particularly difficult.
Comparability	The following suggestions are provided to minimize variables that may affect the comparability protocol: 1. Given that the recombinant reagents have no Factor G pathway, the use of a glucan blocker for the lysate reagent is highly recommended. This will reduce any effects of glucans on the lysate that may alter the comparability test result.	This assumption is wrong and therefore very critical. Under GMP it needs to be demonstrated that any sample preparation including blocking of Glucans is sufficient to demonstrate the method to be free of interfering factors. We would therefore, recommend to use blocking of Glucans as an example for factors that may interfere with detection of endotoxins. Further, it is mandatory to point out that the content of glucans need to be determined by a specialized test before any assumptions can be made that a Glucan Blocker is capable to block interference derived from natural glucans.
Comparability	2. CSEs are not approved by any regional authority nor are they tested by USP laboratories. CSEs are secondary calibration analytes that may be derived from different strains of *Escherichia coli* and formulated differently among reagent suppliers. The use of one manufacturer's CSE with another manufacturer's reagent may result in a different potency determination, which could influence the comparability study outcome (see ⟨1085⟩). It is suggested that comparability studies employ the USP Endotoxin RS for calibration curves and positive product control (PPC) in order to eliminate any effects that an unmatched combination of reagent lot–CSE lot may have on the test result.	CSE is tracible to a WHO reference standard therefore this paragraph does not make sense. As highlighted earlier, the Endotoxin Test is not absolute but a model system. This seems to be forgotten here. We would recommend to continue the recommendation for RSE if authors find this necessary but to delete the entire reference to CSE as this is confusing to the reader.
Comparability	3. An example of acceptance criteria comparing measured endotoxins activity using recombinant reagents and naturally derived lysates might be the following: The measured activity of a sample containing endotoxins using a recombinant reagent should fall within 50%–200% of the measured activity in the same sample tested using naturally occurring lysate as described in ⟨85⟩. A sample calculation is provided below for a single sample where autochthonous endotoxins are measured at 4.7 Endotoxin Units (EU)/mL using the compendial lysate method and 5.3 EU/mL using the recombinant reagent.	We suggest deleting the terms "naturally derived" and "naturally occurring"(lysate) as this does not exist. We request to clarify that that this exercise only makes sense if all interfering factors are eliminated by sample preparation measures. If this is not done it is likely that there is a difference observed even between different compendial reagents. Since authors stated before that lysate reagents will very likely overestimate the example is confusing.
Comparability	If desired, a firm may use a range that is more stringent than the 50%–200% range (e.g., 70%–130%), but the range should be no broader than 50%–200% as prescribed in ⟨85⟩ for the PPC. Scientific discretion should be used to look for patterns in recoveries that may suggest a bias in the test results; for example,	The limits of 50-200% was established for microbiological methods after long debates due to the variability of natural resources. A recommendation to move back to a more stringent definition not only counterproductive but is related to company-specific GMP requirements.

	calculated recoveries clustered at the lower or higher end of the recovery range.	WE would recommend to delete this paragraph.
Comparability	The use of a different statistical model for comparability is up to the user and must be described in validation protocols. For example, if a statistical methodology such as described in ASTM E2935 (*23*) is used, the number of samples and acceptance criteria must be justifiable and consistent with the chosen methodology.	It is confusing as to why USP recommends an outdated ASTM chapter. Why is there no reference to a USP chapter?
Test procedure	For rFC and rCR routine test procedures, ⟨85⟩, *Photometric Quantitative Techniques, Test Procedure* for the endpoint-chromogenic technique and kinetic chromogenic technique, respectively can be utilized.	<85> doesn't make any difference between either kinetic or endpoint, or chromogenic and turbidimetric. It is the same text for all, titled PHOTOMETRIC QUANTITATIVE TECHNIQUES - Preparatory Testing - assurance of criteria for the standard curve.
Interpretation	For rFC and rCR routine test procedures, ⟨85⟩, *Photometric Quantitative Techniques, Test Procedure* for the endpoint-chromogenic technique and kinetic chromogenic technique, respectively can be utilized.	<85> doesn't make any difference between either kinetic or endpoint, or chromogenic and turbidimetric. It is the same text for all, titled PHOTOMETRIC QUANTITATIVE TECHNIQUES - Preparatory Testing - assurance of criteria for the standard curve.
Points to Consider: Supplier Quality	Unlike with their naturally sourced lysate counterparts described in ⟨85⟩, the manufacture, distribution, and use of recombinant reagents have not, at this time, been subject to licensing and inspectional oversight by any regional regulatory authority.	As above, we recommend removing the term "naturally sourced lysate", as this does not exist. Many reagents used for final product release are not regulated, so why are recombinant products being singled out in this regard. Of course any potential user should regard the reagents used for a BET as a critical component of their QC testing. A supplier qualification and a quality agreement should be a given in a GMP environment but once again there is an additional and difficult requirement given to potential users to *'Understand reagent process development with respect to factors that may affect the sensitivity or specificity of the alternative test'.* This is not a requirement for many other final product release tests. As supplier quality is discussed in detail in many guidance documents, it is unnecessary to describe it here. The user should be directed to a reference.
Points to Consider: Supplier Quality	Therefore, it is incumbent on the user of these reagents to assure, through robust and careful supplier management, that the manufacture of recombinant reagents is controlled in a manner such that data from their use are accurate and consistent.	A supplier qualification and a quality agreement should be a given in a GMP environment but once again there is an additional and difficult requirement given to potential users to *'Understand reagent process development with respect to factors that may affect the sensitivity or specificity of the alternative test'.* This is not a requirement for many other final product release tests. Recombinant products described here are not GMP relevant product. Therefore, we believe that a written supplier qualification is sufficient. We recommend to delete this paragraph
Points to Consider: Supplier Quality	Because these reagents are used in the performance of a safety test for critical ingredients, intermediate products, or finished products, it is suggested that suppliers of these reagents be considered critical suppliers in the user's supplier management or vendor certification program, and robust quality agreements be executed.	There seems to be a disconnect to the Introduction that was limiting the chapter to end-product testing. Further, this is again a GMP associated comment which should not be part of this chapter. We suggest that this paragraph be deleted.
Points to Consider: Supplier Quality	Recombinant reagents are not considered to be drugs, devices, or biological products; therefore, their manufacture may not fully comply with standard drug/device/biological current good manufacturing practices (cGMPs). Table 1 provides suggestions on	With the first sentence the entire paragraph below that is only looking into GMP needs for medicinal products must be deleted

	responsibilities of the supplier and user, which may be helpful as guidelines for auditing.	
Points to consider: Supplier quality Table 1 specific comments	General comment	This is not relevant since this is GMP – we suggest that it be deleted. A written supplier qualification is sufficient for reagents outside critical environments The recombinant proteins don't come from the crab - the DNA construct originally came from the crab! There is no reason to outline the design of the product, there are relevant USP chapters which are telling the opposite for reagents not used in GMP processes (one example is USP 1058)
Points to consider: Supplier quality	Quality agreement assuring compliance with the basic tenets of cGMP and no ification of changes in source of materials, processing, and control	Separate quality agreements for components of a bioassay is not recommended. The remaining is part of ISO certification which is provided by the supplier in writing. We suggest that this be deleted
Points to consider: Supplier quality	Understanding reagent process development with respect to factors hat may affect the sensitivity or specificity of the alternative test.	This is not required for Bioassays and there is even worse no scientific evidence that this is relevant. Request for deletion
Points to consider: Supplier quality	Though not required, manufacturers of recombinant endotoxin reagents should consider the submission of a drug master _le or the equivalent depending on the regional au hority.	Lonza agrees with this statement. This is why we submitted a Master File to FDA many years ago.

October 23, 2020

Ms. Leslie Furr
Associate Scientific Liaison
The United States Pharmacopeial Convention, Inc.
12601 Twinbrook Pkwy.
Rockville, MD 20852
[redacted]

Eli Lilly and Company

Lilly Corporate Center
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+1.317.276.2000
www.lilly.com

RE: <1085.1> Use of Recombinant Reagents in the Bacterial Endotoxins Test—Photometric and Fluorometric Methods Using Recombinantly Derived Reagents in PF 46(5)

Dear Ms. Furr (Leslie),

This letter is in response to the draft Guidance posted 01-Sep-2020 in PF 46(5) for a new General Chapter <1085.1>, *Use of Recombinant Reagents in the Bacterial Endotoxins Test—Photometric and Fluorometric Methods Using Recombinantly Derived Reagents.*

Through the Stimuli Article in PF 46(3)[1] and comments submitted in response to the June 2020 Prospectus for the proposed Chapter 1085.1, Lilly has supported inclusion of recombinant factor C (rFC) reagents and methods as acceptable alternatives to the compendia techniques using LAL reagent. This was the original path per the proposed PF 45(5) chapter <85>.

Per our review of the proposed Chapter <1085.1> in PF 46(5), it is apparent that the Expert Committee has the following concerns and potential objections to continuing with the PF 45(5) draft of <85>:

1. Autochthonous endotoxins could go undetected by rFC and pose a unique public health risk.
2. Inadequate published comprehensive data exist that demonstrate comparability of recombinant methods to LAL lysates at assayable levels of endotoxins activity.
3. Because rFC reagents are used in critical tests for patient safety, the quality oversight of such suppliers must ensure consistent functionality and stability of the chosen recombinant components.

We have addressed these concerns in Attachment A.

Lilly holds that as written, the proposed chapter <1085.1> increases barriers to rFC implementation, potentially delays approval of new drug products, and practically prevents the conversion of registered LAL methods to rFC methods.

[1] Stimuli Article-Application of rFC Reagent for the Detection of Bacterial Endotoxins … Comparability to Limulus Amebocyte Lysate

Please consider making the following changes, and reference additional discussion in Attachment A:

- RE: Duplicative validation requirements: Remove from the proposed chapter <1085.1> the proposed occurrences of additional validation proof beyond what is already stated in the FDA Guideline on Analytical Procedures and Methods Validation for Drugs and Biologics[2] and <1225>, specifically the proposed Comparability section and guidance.
- RE: Out of Scope Quality System Guidance: Remove from the proposed chapter <1085.1> the proposed Points to Consider section on supplier quality as that is out of scope of public health standards

The USP should engage all significant stakeholders and drive to a General Test as originally planned, and as quickly as possible, based on deep technical understanding of the underlying biotechnology science, and as supported by both the peer-reviewed literature, including those in Attachment B and the confidential data provided herein Attachment C. We encourage the USP to provide flexibility and to support bringing new products to patients in a timely manner by representing the use of rFC within a general test chapter.

Note: We support the sharing of comments per this letter, Attachment A and Attachment B, but do not support public distribution of Attachment C.

If you have questions or other concerns, please contact us.

ELI LILLY AND COMPANY

[redacted] [redacted]

Attachment A – Supplemental Rationale for Lilly's Recommendations Re: <1085.1> in PF 46(5)

Attachment B - A Review of Existing Literature on the Use of Recombinant Factor C (rFC) for Bacterial Endotoxin Testing

Attachment C - Confidential Lilly Data Re: BET by rFC and LAL – Not for External Distribution (Attachment provided in separate document)

[2] https://www.fda.gov/files/drugs/published/Analytical-Procedures-and-Methods-Validation-for-Drugs-and-Biologics.pdf

Attachment A – Supplemental Rationale for Lilly's Recommendations Re: <1085.1> in PF 46(5)

Detection of Autochthonous Endotoxins at Assayable Levels

The potential risks posed by autochthonous endotoxins, for example those that might be introduced by improperly purified water, might also result in atypical sterility and bioburden testing results. There is no expectation when validating an alternative rapid sterility or bioburden methods according to <1223> that the end-user provide comparability data using samples containing autochthonous organisms. Instead, compendia sterility, bioburden and endotoxin methods use common, traceable public standard culture collection organisms or reference standard endotoxin to demonstrate that the respective method is valid for intended purpose. Sterility, bioburden and endotoxin methods are employed as a final check that the sterility/control strategy is successful and are only predictive models to make claims regarding product quality. We hold that it is unnecessary to add this type of validation requirement to ensure that rFC reagents and methods are suitable for their intended use.

Additionally, the suggestion to use low grade pharmaceutical water, or any sample potentially containing beta glucans, for comparability purposes is inappropriate for the following reasons:

- Uncharacterized beta glucans, coming in from municipal water sources, are present in low grade pharmaceutical waters.
- Lilly does not maintain endotoxin specifications nor routinely tests low grade pharmaceutical waters. Lilly does allow significant bioburden (50,000 CFU/100 mL) for such for low-grade water. Endotoxin removal is claimed in subsequent ultrafiltration or distillation steps in the generation of high-grade pharmaceutical waters such as Water for Injection.
- The <1085.1> draft acknowledges that beta glucans cause false positive results in the BET, and LAL can synergistically overpredict BET results in the presence of endotoxin, which is a very likely explanation for publicly-claimed disparities in rFC vs. LAL data for samples containing uncharacterized beta glucans.
- Data exists demonstrating that commercial beta glucan blocking buffers do not always completely block all beta glucan (specificity). To say the use of blocking buffers in <1085.1> "…will reduce any effects of glucans…" is not true.
- Beta glucan characterization in such samples would require complex mass spectrometry, which would be difficult to do at scale.

Published comprehensive data exist that demonstrate comparability of recombinant methods to LAL lysates

Current peer-reviewed literature exists covering a broad specificity of rFC toward a wide range and diversity of endotoxins (Attachment B provides a number of these). In August 2020, the PDA published a review by a panel of industry, regulatory and compendia bacterial endotoxins subject matter experts of the existing literature and concluded that rFC is comparable to LAL. Note the following numbers derived from the encompassing literature set per Attachment B:

- 213 different relevant pharmaceutical products have been reported using rFC in 8 broad categories including:

- large molecule/peptide drug product/drug substance
- small molecule drug product/API
- vaccine
- excipients/raw materials
- container closure component
- buffers/pharma grade waters
- clinical samples
- plant extract

- At least 1087 unique samples containing environmental (real world) endotoxin were reported using rFC, most with head-to-head corresponding LAL data.
- Nearly all the cited literature establishes comparability, or demonstrates that rFC is equivalent or superior to LAL

Regarding assayable levels of endotoxins, the following references specifically contain data that quantitated environmental endotoxin head-to-head rFC:LAL:

Author	Title
Alwis, K. U., and D. K. Milton	Recombinant factor C assay for measuring endotoxin in house dust: comparison with LAL, and (1–3)- -Dglucans.
Thorne et al	Evaluation of the Limulus Amebocyte Lysate and Recombinant Factor C Assays for Assessment of Airborne Endotoxin
McKenzie et al	Evaluation of lot-to-lot repeatability and effect of assay media choice in the recombinant Factor C assay
Chen and Mozier	Comparison of Limulus amebocyte lysate test methods for endotoxin measurement in protein solutions
Schwarz et al	Residual Endotoxin Contaminations in Recombinant Proteins Are Sufficient to Activate Human CD1c+ Dendritic Cells. Schwarz, H et al. 12, December 5, 2014, PLoS ONE, Vol. 9. DOI:10.1371/journal.pone.0113840
Bolden and Smith	Application of Recombinant Factor C Reagent for the Detection of Bacterial Endotoxins in Pharmaceutical Products
Kikuchi et al	Collaborative Study on the Bacterial Endotoxins Test Using Recombinant Factor C-based Procedure for Detection of Lipopolysaccharides Part 1
Marius et al	Comparison of LAL and recombinant Factor C assays for endotoxin detection in four human vaccines with complex matrices
Bolden	Application of Recombinant Factor C Reagent for the Detection of Bacterial Endotoxins in Pharmaceutical Products and Comparability to LAL

Based on fundamental modern recombinant biotechnology principles, rFC is equivalent to LAL in that the recombinant Factor C protein is the clone of the horseshoe crab natural Factor C, which is the bacterial endotoxins biosensor. The references to historical requirements of moving from the Rabbit Pyrogen Test to LAL are not applicable. The rFC assay employs the Factor C biosensor and uses the same Reference Standard Endotoxin calibrator for the same assay readout: the detection of endotoxin expressed in Endotoxin Units (EU).

Quality Oversight of rFC Reagents Suppliers

We concur that rFC reagents are used in critical tests for patient safety, and that it is the responsibility of the user to maintain control strategies that ensure their consistent functionality and stability. Regulatory guidelines exist that provide the 'cGMPs' through established regulatory agencies (e.g., EMA, FDA, ICH). Quality system content is under the purview of a firm

per cGMP law, therefore it is out of place to specify quality system practices in a pharmacopoeia method guidance chapter.

We do not see a benefit with the USP providing replicate standards that mirror already accepted guidelines. Additionally, the content in Table 1 of the proposed <1085.1> could be applicable to many reagents used in the performance of a safety test for critical ingredients, intermediate products, or finished products. These reagents are neither licensed or inspected by any health authority. These include sterility and microbiology test media, QPCR reagents for viral and mycoplasma DNA detection, microbial identification libraries, etc.

Additional Recommendations:

Duplicative validation requirements

The proposed comparability testing per draft <1085.1> requires an undue burden of proof regarding comparability which is not consistent with <1225> and equivalent global pharmacopoeia chapters, or the FDA Guidance. Please consider the following:

Procedural, Regulatory and Principle

- References to <1223> are not applicable as <85> resides under Biological Tests and Assays, and not Microbiological Tests and Assays in the USP. Therefore <1225> is the guiding principle which is affirmed in the FDA Guidance.
- Regulatory authorities around the world have reviewed Lilly marketing applications and have approved Emgality® in multiple markets (over 30, including in the U.S., Europe, Australia, Brazil, Canada, Israel, Kuwait, Lebanon, South Korea, Switzerland, Taiwan, United Arab Emirates). This approval, and discussions regarding future products have demonstrated that global regulatory scientists are generally favorable toward the use of rFC.

External Data

- Kevin Williams successfully repeated a classical endotoxin/beta glucan experiment described by Rolansky and Novitsky 1991 demonstrating overprediction of LAL in environmental water containing beta glucans. Post-treatment with beta glucan blocking buffer (which incompletely blocked the activity), followed by glucanase and cellulase, brought the endotoxin level to that which was reported using rFC, which is inherently not susceptible to the glucan interference compared to LAL.

Internal Data

- Lilly has over 5 years of internal experience across a diverse set of 80+ pharmaceutical products and 60,000+ samples tested using rFC across multiple sites.
- Five internal and confidential comparability data sets using relevant pharmaceutical products is provided in Attachment C. These data sets clearly demonstrate rFC-to-LAL comparability and the importance of beta glucan when evaluating comparability between the two methods.

Attachment B - A Review of Existing Literature on the Use of Recombinant Factor C (rFC) for Bacterial Endotoxin Testing

Author	Title	Journal	Year	Vol	Pages
Alwis and Milton	Recombinant factor C assay for measuring endotoxin in house dust: comparison with LAL, and (1–3)- -Dglucans.	Am. J. Ind. Med.	2006	49	296–300
Thorne et al	Evaluation of the Limulus Amebocyte Lysate and Recombinant Factor C Assays for Assessment of Airborne Endotoxin	Appl Environ Micro	2010	76(15)	4988-4995
Loverock et al	A Recombinant Factor C Procedure for the Detection of Gram-negative Bacterial Endotoxin	USP PF	2010	36 (1)	321-329
McKenzie et al	Evaluation of lot-to-lot repeatability and effect of assay media choice in the recombinant Factor C assay	J Environ Monit	2011	13(6)	1739-1745
Chen and Mozier	Comparison of Limulus amebocyte lysate test methods for endotoxin measurement in protein solutions	J Pharm Biomed Anal	2013	80	180-185
Schwarz et al	Residual Endotoxin Contaminations in Recombinant Proteins Are Sufficient to Activate Human CD1c+ Dendritic Cells	PLoS ONE	2014	9(12)	N/A
Abate et al	Evaluation of recombinant factor C assay for the detection of divergent lipopolysaccharide structural species and comparison with Limulus amebocyte lysate-based assays and a human monocyte activity assay	J Med Microbiol	2017	66(7)	888-97
Bolden and Smith	Application of Recombinant Factor C Reagent for the Detection of Bacterial Endotoxins in Pharmaceutical Products	J Pharm Sci Technol	2017	71(5)	405-412
Bolden et al	Results of a harmonized endotoxin recovery study protocol evaluation by 14 BioPhorum Operations Group (BPOG) member companies	Biologicals	2017	48	74-81
Kikuchi et al	Collaborative Study on the Bacterial Endotoxins Test Using Recombinant Factor C-based Procedure for Detection of Lipopolysaccharides Part 1	Pharm & Med Device reg Science	2017	vol 40(4)	252-260
Kikuchi et al	Collaborative Study on the Bacterial Endotoxins Test Using Recombinant Factor C-based Procedure for Detection of Lipopolysaccharides Part 2	Pharm & Med Device reg Science	2018	vol 49(10)	708-718
Muroi et al	Application of a recombinant three-factor chromogenic reagent, PyroSmart, for bacterial endotoxins test filed in the Pharmacopeias	Biol. Pharm. Bull.	2019	DOI:10.1248/bpb.b19-00517	
Strachan et al	Subgingival lipid A profile and endotoxin activity in periodontal health and disease	Clinical Oral Investigations	2019	23(9)	3527-3534
Pei Yusheng et al	Study on the Applicability of Recombinant Factor C Method for Detection of Bacterial Endotoxin	China Pharmaceuticals	2019	28 (7) DOI 10.3969 / j. issn. 1006-4931.2019.07.006	
Marius et al	Comparison of LAL and recombinant Factor C assays for endotoxin detection in four human vaccines with complex matrices	J Pharm Sci Technol	2020	DOI:10.5731/pdajpst.2019.010389	
Bolden	Application of Recombinant Factor C Reagent for the Detection of Bacterial Endotoxins in Pharmaceutical Products and Comparability to LAL	USP PF	2020	46(3)	N/A
Piehler et al	Comparison of LAL and rFC assays - Participation in a Proficiency Test Program between 2014 and 2019	Microorganisms	2020	8(3)	418 doi: 10.3390/microorganisms8030418.

Pharmacopeia Draft Commenting Summary	
Chapter No., Title	**<1085.1> USE OF RECOMBINANT REAGENTS IN THE BACTERIAL ENDOTOXINS TEST—PHOTOMETRIC AND FLUOROMETRIC METHODS USING RECOMBINANTLY DERIVED REAGENTS**
Publication Name & version	**PF 46.5**

Section # or Name	Comment	Proposed changes	Rationale
General Comment for USP<1085.1>	*USP<1223> is referenced in the document. However, Bacterial Endotoxin Test (BET) is not a microbiological test.*	*When USP <1225> is available for revision, the proposal is to include reference to User Requirement Specification and Comparability- for comparability of recombinant reagents*	*USP<1225> is for biological tests and USP<1223> is for microbiological tests. USP<85> BET is a biological test.*
Briefing	*Other USP chapters, principally Validation of Alternative Microbiological Methods <1223>, Validation of Compendial Procedures <1225>*	*Remove Reference: Validation of Alternative Microbiological Methods <1223>*	*USP<1225> is for biological tests. USP<1223> is for microbiological tests. USP<85> is a biological test.*
Briefing	*Photometric and Fluorometric Methods*	*Delete "Photometric and": ~~Photometric and~~ Fluorometric Methods*	*To my knowledge the photometric method (Pyrosmart Seikagaku) is not (broadly ?) available to the US market until today and the Charles River photometric method is not even finished.* *Pharmacopeias should not recommend or reference methods/technologies not available on the market or fairly unknown.*
Validation of Alternative Methods	*Comparability: therefore demonstrate equivalency of results per 〈1223〉. (For further information, see Comparability.)*	*Remove <1223> Reference*	*USP<1225> is for biological tests. USP<1223> is for microbiological tests. USP<85> is a biological test.*

Section # or Name	Comment	Proposed changes	3 Rationale
Comparability	*CSEs or RSEs may be used in addition to autochthonous endotoxins if required by a regional regulatory authority.*	*CSEs or RSEs may be used. Autochthonous endotoxins may be used, if available, as supportive data.*	*In a very well maintained water system and clean feed water source, it may be difficult to locate relevant autochthonous endotoxins.*
Comparability	*Thus, an appropriate analyte may be water taken from the upstream water for injection (WFI) purification stream after the carbon filters, for example, or in some cases deionized water may be used.*	*Specify if using one water sample that contains autochthonous endotoxins is enough for comparability studies or if the expectation is that more than one water sample will be compared.*	*If locating one appropriate/relevant sample is difficult, more than one will not be possible for small, well maintained water systems*
Comparability	*The use of "autochthonous endotoxins" in the comparability section*	*There is no standardization of autochthonous endotoxins. Therefore, the outcome of the results is difficult to interpret.*	*There are no publications available on the use of autochthonous endotoxins. The term "endotoxins from autochthonous manufacturing sources" has not been used in relevant scientific publications.* *There is no published data which support the unique requirement that an analytical methods used for safety testing must demonstrate comparability using naturally contaminated sample material.*
Comparability	*The use of "autochthonous endotoxins" in the comparability section*	*Delete the requirement to use "autochthonous endotoxins"*	*In general, the proposal to use contaminated batches (very rare) or batches contaminated with non-identified and non-characterized "autochthonous contaminants" seems exclusive for rFC, this is not requested for other relevant safety tests e.g. (alternative) sterility tests.*
Points to Consider: Supplier Quality	*Whole section*	*Delete whole section*	*First time seeing common GMP requirements (here: Supplier and User Responsibilities) in a Pharmacopeial Monograph.* *The requirements are very one-sided focused on rFC, e.g. "Documentation of reagent formulation": the formulation of the LAL is unknown (and varies from manufacturer to manufacturer).*
Points to Consider: Supplier Quality	*[...], manufacturers of recombinant endotoxin reagents should consider the submission of a drug master file or the equivalent depending on the regional authority*	*Delete whole section*	*Even if the manufacturer-specific formulation would have been filed in a DMF, this information is not accessible to customers outside the USA.* *Equivalent files are not available all over the globe.*

Roche appreciates USP’s consideration of these comments and we also welcome the opportunity to discuss any of these points further.

If you have questions or other concerns, please feel free to contact me via email :[redacted]

Sincerely

[redacted]

From:	Pharmacopeial Forum
To:	Leslie Furr; lynn_best@baxter.com
Subject:	<1085.1> Use of Recombinant Reagents in the Bacterial Endotoxins Test - Photometric and Fluorometric Methods Using Recombinantly Derived Reagents - PF 46(5) - Comment- <1085>.1
Date:	Friday, November 13, 2020 10:56:12 AM
Attachments:	11 13 20 Comments 1085.1.docx

Sender Details:

First Name:	Lynn	**Last Name:**	Best
Company:	b17ecad541dc4a2e8f009b750a514c9ff28ac35b	**Email:**	lynn_best@baxter.com

Metadata:

Time:	11/13/2020 03:55 PM UTC	**Remote Server:**	online.usppf.com
No of Attachments:	1	**Publication:**	PF 46-5
Doc Id:	GUID-8AA2F02F-6FD6-4248-A07B-C0669C2E8DFA_10101_en-US	**GUID:**	GUID-8AA2F02F-6FD6-4248-A07B-C0669C2E8DFA
Version:	10101	**Language:**	en-US

Original Message:

Add the following text as indicated in red in the Attachment below:

Figure 1. Natural lysate zymogen protease cascade (*4*).

Recombinant bacterial endotoxins test (BET) reagents proposed as alternatives to naturally sourced *Limulus* amebocyte lysate (LAL) or *Tachypleus* amebocyte lysate (TAL) contain one or more recombinant zymogen protease(s) cloned from one or more constituent zymogen elements of the natural cascade. By design, recombinant reagents lack the alternative Factor G pathway where the presence of β-D-glucan can activate Factor G, which in turn can act as a non-endotoxin-specific activator of the proclotting enzyme. When a sample contains sufficient β-D-glucan, the presence of the Factor G pathway in natural lysate can result in an overestimation of endotoxins activity (*5–6*).

From:	Pharmacopeial Forum
To:	Leslie Furr; ingo.spreitzer@pei.de
Subject:	<1085.1> Use of Recombinant Reagents in the Bacterial Endotoxins Test - Photometric and Fluorometric Methods Using Recombinantly Derived Reagents - PF 46(5) - discard 1085.1 draft
Date:	Monday, November 23, 2020 4:55:05 AM
Attachments:	20201123 comments- Draft USP 1085.1 Ingo Spreitzer.docx

Sender Details:

First Name:	Ingo	**Last Name:**	Spreitzer
Company:	e943fc7a9e78a305c32802b868d34358c15a71d7	**Email:**	ingo.spreitzer@pei.de

Metadata:

Time:	11/23/2020 09:54 AM UTC	**Remote Server:**	online.usppf.com
No of Attachments:	1	**Publication:**	PF 46-5
Doc Id:	GUID-8AA2F02F-6FD6-4248-A07B-C0669C2E8DFA_10101_en-US	**GUID:**	GUID-8AA2F02F-6FD6-4248-A07B-C0669C2E8DFA
Version:	10101	**Language:**	en-US

Original Message:

```
The proposed 1085.1 is worsening the situation for rFC for unknown
reasons. Description of Spiking experiments to my point of view wrong
(BET-result is set as 100%, return of the "golden Standard").
See attached file for comments
```

United States Pharmacopeia
⟨ 1085.1⟩ Use of Recombinant Reagents in the Bacterial Endotoxins Test—
Photometric and Fluorometric Methods Using Recombinantly Derived Reagents
03-Sep-2020

General Comments

General Comments	**Rationale**
I feel USP wants to suppress rFC as long as needed for unknown reasons.	USP introduces exclusive hurdles for rFC compared to e.g. alternative sterility tests

Specific Comments to the Text

Section	Current Text	Proposed Change	Rationale
Briefing	Photometric and Fluorometric Methods	~~Photometric and~~ Fluorometric Methods	To my knowledge the Photometric Methods (PyroSmart, PyroSmart NextGen, Seikagaku) are not broadly available to the US market until today. **Compared to rFC the independent data amount is scarce. Pharmacopeias do not recommend methods not available or fairly unknown**. The Charles River photometric method is not even finished.
	Validation of Alternative Microbiological Methods ⟨1223⟩	~~*Validation of Alternative Microbiological Methods* ⟨1223⟩~~	I believe that BET and consequently rFC do not belong to <1223>
Comparability	Historically, prior to the acceptance of the LAL method as comparable to the rabbit pyrogen test it replaced, comprehensive studies were performed to assure that the LAL method could provide equivalent (or better) product quality decisions (*20*). Currently, although data are available on suitability (inhibition/enhancement) testing using recombinant reagents, comprehensive data demonstrating comparability of recombinant methods to LAL lysates in compendial articles containing *assayable levels* of endotoxins activity from autochthonous endotoxins are not available or have not been published in the public domain. Therefore, until such data become available it is up to each stakeholder who wishes to qualify a recombinant BET to undertake appropriate comparability trials.	Each stakeholder who wishes to qualify a recombinant BET has to perform a full test validation and the product specific validation	Common regulatory approach for alternative methods. Regulators will decide if data are sufficient. The USP-remark on lack of data on "autochthonous Endotoxins" in compendial articles is wrong to our point of view, see PDA article doi:10.5731/pdajpst.2020.012187

	an appropriate analyte may be water taken from the upstream water for injection (WFI) purification stream after the carbon filters, for example, or in some cases deionized water may be used.	an appropriate analyte might be the product spiked with RSE, probably in addition to the use of NOE (commercial preparation)	Unsterile water samples containing unknown (autochthonous) endotoxins + Limulus reactive material (LAM) are not appropriate for pharmacopeial proposals (standardization?), whereas products (or WFI for assay validation) spiked with RSE and probably NOE (commercial preparation) are. Doing the comparability by calculating the rFC-"recovery" of unknown (naturally contaminated water) samples by just setting the BET-result to 100% is wrong. Sample has to be spiked with a known amount of Endotoxin, then rFC and BET-results have to be judged against this theoretical value. The use of home-grown or unknown home-derived NOE is scientifically relevant, but contradicts the nature of Pharmacopeias (standardization, comparability). I believe that the unpublished data from Charles River on CF- and DI-water are misinterpreted by Charles River and the USP as a sign of inferiority of rFC. Kevin Williams from BioMerieux has recently published interesting data on "natural water" in the American Pharmaceutical Review July/August 2020, p. 104-, which explicitly

Section	Current Text	Proposed Change	Rationale
			questions the superiority of BET on CF- and DI-water. The USP-Micro Expert Committee uses the same journal for publication, so they might want to read this.
			In general, the proposal to use contaminated batches (very rare) or batches contaminated with non-identified autochthonous contaminants seems exclusive for rFC, this is not requested for other relevant safety tests.like alternative sterility tests

United States Pharmacopeia
⟨ 1085.1⟩ Use of Recombinant Reagents in the Bacterial Endotoxins Test—
Photometric and Fluorometric Methods Using Recombinantly Derived Reagents
03-Sep-2020

Section	Current Text	Proposed Change	Rationale
POINTS TO CONSIDER: SUPPLIER QUALITY	Whole section	Delete whole section	First time that I see common GMP requirements in a Pharmacopeial Monograph. If you do sterility testing you are not obliged by the USP to qualify your supplier of culture media like it is now imposed for rFC. USP is taking a very strange direction, I wonder how the PDG sees this development. I do not think that there are any drug master files on the BET-Lysates or the Control Standard Endotoxins. If so, it would be much easier to understand the relevant differences between different Lysate manufacturers and even lysate batches from the same manufacturer (and the differences between the CSE´s).

United States Pharmacopeia

⟨ 1085.1⟩ Use of Recombinant Reagents in the Bacterial Endotoxins Test—
Photometric and Fluorometric Methods Using Recombinantly Derived Reagents

03-Sep-2020

Section	Current Text	Proposed Change	Rationale

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Germany

November 24, 2020

Leslie Furr
12601 Twinbrook Parkway
Rockville, MD 20852-1790, USA

Reference to Correspondence Number – C273155
Proposed <1085.1> Use of Recombinant Reagents in the Bacterial Endotoxins Test – Photometric and Fluorometric Methods Using Recombinantly Derived Reagents – USP PF 46(5)

Dear Ms. Furr:

PDA is pleased to have the opportunity to provide comments on the proposed USP Informational Chapter <1085.1>, released for public comment on September 1, 2020. We recognize that the purpose of the proposed chapter is to provide guidance on the qualification and validation of recombinant reagents as alternatives to naturally sourced reagents from horseshoe crab hemolymph for the purposes of quantitating endotoxin activity.

Our comments were prepared by an international group of expert volunteers with experience in drug product regulation, development, and manufacture specifically related to endotoxin testing, including the drafting of pharmacopeial guidance. The following pages present some concerns with the proposed chapter overall, as well as a number of technical comments. The specific technical comments are organized by the draft's section headings.

Of particular concern to the group was:

1) The inclusion of additional hurdles for the use of recombinant reagents for endotoxin detection compared to other USP guidance on the use of alternative methods
2) The inclusion of procedures for ensuring supplier quality as this is not specific for recombinant reagents and these considerations are not part of a description provided by USP for other, similar reagents.

PDA is a non-profit, international, professional association of more than 10,000 individual member scientists having an interest in the fields of pharmaceutical, biological, and device manufacturing and quality. These comments were prepared by a committee of experts with experience in the practice of pharmacy and pharmaceutical manufacturing including members representing our Board of Directors, our Science Advisory Board, and our Regulatory Affairs and Quality Advisory Board.

If there are any questions, please do not hesitate to contact me.

Sincerely,

Richard Johnson
President, PDA

CC: Glenn Wright, PDA; Joshua Eaton, PDA

PDA Comment to United States Pharmacopeia
⟨1085.1⟩ Use of Recombinant Reagents in the Bacterial Endotoxins Test –
Photometric and Fluorometric Methods Using Recombinantly Derived Reagents
01-Sep-2020

General Comments	Rationale
This proposed chapter seems to be attempting to dissuade the use of rFC	USP introduces exclusive hurdles for rFC compared to other USP guidance (e.g., alternative sterility tests)
The USP should engage all stakeholders to develop a General Test as originally planned.	There is extensive technical understanding of the underlying biotechnology science which is supported by current peer-reviewed literature produced by experts in the field. The drafting committee needs to consider and incorporate the most recent, relevant, peer-reviewed data (see PDA article doi:10.5731/pdajpst.2020.012187 for examples)
Further international dialogue on this topic should be a priority given the large volume of parenterals entering the market from vaccine needs and other products.	The rationale for why USP is diverging from other pharmacopeia needs to be resolved. A harmonized test expedites drug development and approval

Specific Comments to the Text

Section	Current Text	Proposed Change	Rationale
Briefing	……..requires demonstration of comparability based on criteria recommended in this chapter proposal and other *USP* chapters, principally *Validation of Alternative Microbiological Methods* ⟨1223⟩, *Validation of Compendial Procedures* ⟨1225⟩, and *Guidelines on the Endotoxins Test* ⟨1085⟩ as noted.	Remove references to <1223> throughout the document	Endotoxin is not a microbiology test (Reference the FDA guidance from 2012)
Briefing	"..preapproval…"	Change to 'approval'	FDA would review as part of a filing. Possibly reference a suggestion to discuss with FDA. The document shouldn't speak to what other health authorities would require. Seems to ignore EP 2.6.32.
Briefing	Photometric and Fluorometric Methods	Fluorometric Methods	To our knowledge the Photometric Method (Pyrosmart, Seikagaku) is not broadly available to the US market at this time. Compared to rFC the availability of end-user data is limited. Pharmacopeias do not recommend methods not available or fairly unknown. The Charles River photometric method is not yet available.
Background	(kinetic turbidimetric assay)	(turbidimetric assay)	Both endpoint and kinetic assay are in use

PDA Comment to United States Pharmacopeia
⟨1085.1⟩ Use of Recombinant Reagents in the Bacterial Endotoxins Test –
Photometric and Fluorometric Methods Using Recombinantly Derived Reagents
01-Sep-2020

Section	Current Text	Proposed Change	Rationale
Background	"Recombinant Cascade Reagents"	Confirm that this is what the reagent supplier calls them.	Harmonize verbiage
Background	A Species of horseshoe crab	From either one of two species	Stylistic
Background	Fig 2, (rFC cascade) Fig 3 (Recombinant Cascade Chromogenic Reaction)	Harmonize the two figure titles.	Stylistic change
Validation of alternative methods	3. Comparability: …. comparability of the recombinant reagents to naturally sourced lysate using endotoxins from autochthonous manufacturing sources is of particular importance.	Rewrite section and delete term "endotoxins from autochthonous manufacturing sources"	The term "endotoxins from autochthonous manufacturing sources" has not been used in the relevant literature and needs further explanation. There are published data which demonstrates comparability for rFC and LAL using environmental endotoxins (Bolden et al., 2020. PDA Journal of Pharmaceutical Science and Technology August 2020, pdajpst.2020.012187). It is not sufficiently justified to request additional comparability data using environmental endotoxins for each product-specific validation.
Validation of alternative methods	Prior to validation of an endotoxins test using recombinant reagents, a user requirement specification (URS) should be produced per Validation of Alternative Microbiological Methods ⟨1223⟩.	Remove reference to <1223>	Test for bacterial endotoxins is not regarded as a microbiological method and thus <1223> does not apply.
Validation of Alternative Methods (Comparability)	"demonstrate equivalency of results"	Remove this statement	Not in harmony with FDA 2012 Guidance which specified equivalent or better results, or alignment with <1225>.

PDA Comment to United States Pharmacopeia
⟨1085.1⟩ Use of Recombinant Reagents in the Bacterial Endotoxins Test –
Photometric and Fluorometric Methods Using Recombinantly Derived Reagents
01-Sep-2020

Section	Current Text	Proposed Change	Rationale
Validation of Alternative Methods	"Therefore, it is incumbent on the user of these reagents to assure that the manufacture…"	Remove most of this sentence	It imposes a greater requirement than other reagents used throughout the chapters in the USP. In a typical supplier audit, it would not be possible to assure all of the specified attributes. It is inappropriate for USP to request that a supplier audit should be conducted.
Validation of Alternative Methods	Unless otherwise indicated in the monograph	Remove	This is general knowledge and probably doesn't need to be stated here.
Preparatory Tests and General Notes	Apparatus: Reference ⟨85⟩. For fluorometric tests, qualify instruments per Analytical Instrument Qualification ⟨1058⟩ and calibrate the instrument according to the manufacturer's instructions.	Remove directions on how to qualify the instrument. <1085> is an informational chapter	<1085> is an informational chapter and, while the information in the chapter may be good information to have, it is not appropriate to include it in this USP standard.
Preparatory Tests and General Notes	Reagents and test solutions: Reference ⟨85⟩ except for recombinant reagents. For those, follow the manufacturer's instructions for storage, reconstitution, and use.	Remove	This is a general expectation and including it here doesn't seem to serve a purpose.
Comparability	Historically, prior to the acceptance of the LAL method as comparable to the rabbit pyrogen test it replaced, comprehensive studies were performed to assure that the LAL method could provide equivalent (or better) product quality decisions (*20*).	Remove this reference	References to historical requirements of moving from the Rabbit Pyrogen Test to LAL are not applicable with respect to the use of biotechnology to clone the natural protein and using the same Reference Standard Endotoxin calibrator for the same assay readout: i.e., the detection of endotoxin expressed in Endotoxin Units (EU).

PDA Comment to United States Pharmacopeia
⟨1085.1⟩ Use of Recombinant Reagents in the Bacterial Endotoxins Test –
Photometric and Fluorometric Methods Using Recombinantly Derived Reagents
01-Sep-2020

Section	Current Text	Proposed Change	Rationale
Comparability	Currently, although data are available on suitability (inhibition/enhancement) testing using recombinant reagents, comprehensive data demonstrating comparability of recombinant methods to LAL lysates in compendial articles containing *assayable levels* of endotoxins activity from autochthonous endotoxins are not available or have not been published in the public domain. Therefore, until such data become available it is up to each stakeholder who wishes to qualify a recombinant BET to undertake appropriate comparability trials.	Remove this reference	The USP-remark on lack of data on "autochthonous Endotoxins" is inaccurate, see PDA article doi:10.5731/pdajpst.2020.012187 Nearly all the cited literature establishes comparability or demonstrates that rFC is equivalent or superior to LAL. At least 1,087 unique samples containing environmental (real world) endotoxin were reported using rFC: most with head-to-head corresponding LAL data. 213 different relevant pharmaceutical products have been reported as using rFC (most with head-to-head corresponding LAL data) in 8 broad categories including: - large molecule/peptide drug product/drug substance - container closure components - small molecule drug product/API - buffers/pharma grade waters - vaccines - clinical samples - excipients/raw materials - plant extracts The European and Chinese Pharmacopoeias recognize the use of rFC for compendia purposes. Regulators will decide if data are sufficient, which is common regulatory approach for alternative methods.

Section	Current Text	Proposed Change	Rationale
Comparability	an appropriate analyte may be water taken from the upstream water for injection (WFI) purification stream after the carbon filters, for example, or in some cases deionized water may be used.	an appropriate analyte might be the product spiked with RSE or CSE	Unsterile water samples containing unknown contaminations including (autochthonous) endotoxins and beta-glucans are not appropriate for pharmacopeial proposals (standardization?), whereas products (or WFI for assay validation) spiked with RSE or CSE are. In general, the proposal to use contaminated batches (very rare) or batches contaminated with non-identified autochthonous contaminants seems exclusive for rFC, this is not requested for other relevant safety tests, like alternative sterility tests.
Comparability	Given that the recombinant reagents have no Factor G pathway, the use of a glucan blocker for the lysate reagent is highly recommended. This will reduce any effects of glucans on the lysate that may alter the comparability test result.	Reword to “This may reduce the effects of glucans on the lysate that may alter the comparability test result.”	Beta glucan blocking buffers do not always completely block all beta glucan (specificity). To say the use of blocking buffers in <1085.1> “…will reduce any effects of glucans…” is not true. (Roslansky and Novitsky, Sensitivity of Limulus amebocyte lysate (LAL) to LAL-reactive glucans, Journal of Clinical Microbiology, Nov. 1991, p. 2477-2483.) This seems self-evident; however it is not clear the way that this is written that one would need the beta glucan buffer for the <85> test and not for the rFC assay.

PDA Comment to United States Pharmacopeia
⟨1085.1⟩ Use of Recombinant Reagents in the Bacterial Endotoxins Test –
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01-Sep-2020

Section	Current Text	Proposed Change	Rationale
Comparability	CSEs are not approved by any regional authority nor are they tested by USP laboratories. CSEs are secondary calibration analytes that may be derived from different strains of Escherichia coli and formulated differently among reagent suppliers. The use of one manufacturer's CSE with another manufacturer's reagent may result in a different potency determination, which could influence the comparability study outcome (see ⟨1085⟩). It is suggested that comparability studies employ the USP Endotoxin RS for calibration curves and positive product control (PPC) in order to eliminate any effects that an unmatched combination of reagent lot–CSE lot may have on the test result.	Clarify this statement	rFC suppliers provide matched endotoxin standards (CSE) paired to specific reagent batches and are calibrated to the RSE. It might be appropriate to use CSEs in a comparability study that are matched to a specific supplier (or RSE), but we agree it would be inappropriate to use a BMX CSE with a Lonza rFC per the stated example. Please clarify the statement as such.
Comparability	Relative Recovery calculation	Remove this whole statement and calculation reference	This is not correct. For example, if the acceptance criteria are both 50 to 200% recovery, if you spiked 5 EU in the product, and the assay was 10 EU, then it would be acceptable. If you then assayed by rFC and got 2.5 EU, this would be an acceptable result. But the calculation here would give 2.5/10 *100 = 25% recovery
Comparability	Historically, the source of endotoxins entering manufacturing processes has most often been aquatic Gram-negative bacteria colonizing water systems	Include additional, more recent references	There has been a lot of relevant history in the last 75+ years.
Points to Consider: Supplier Quality	Entire section	Remove	Procedure for ensuring supplier quality should not be included. It is not specific for recombinant reagents and it is not part of description for other similar reagents in USP. General GMP requirements should not be included in the chapter.

charles river

23rd November 2020

To Whom It May Concern

RE: Charles River Microbial Solutions Response to Proposed USP Chapter 1085.1: Use of Recombinant Reagents in the Bacterial Endotoxins Test – Photometric and Fluorometric Methods Using Recombinantly Derived Reagents

Correspondence Number: C273155

Regarding:

Page 1, Line 1 – '*Bacterial Endotoxins Test* ⟨85⟩ is a harmonized analytical procedure…'

> **Comment:** Bacterial Endotoxin Testing should not be listed as an analytical procedure. Endotoxin Detection reagents are not analytical chemistry reagents designed to quantify exact amounts of endotoxin in a sample. They are alternative pyrogen tests which are used to ensure injectable drugs are screened for dangerous levels of Gram-negative pyrogens prior to release and distribution to patients.

Regarding:

Page 1, **Briefing,** Lines 3 and 4 – '… quantitating endotoxins activity in injectable drugs, biologics, and medical device extracts where applicable.'

and

Page 4, **Points to Consider: Supplier Quality**, Line 4 – 'Because these reagents are used in the performance of a safety test for critical ingredients, intermediate products, or finished products…'

> **Comment:** It is important to note that intermediate products and critical ingredients are under this informational chapter for recombinant reagents. By including Water for Injection (WFI) as an example for critical ingredients, and injectable drugs, biologics, and medical device extracts as examples for finished products, the points to consider become more harmonized.

Regarding:

Page 4, **Comparability**, Lines 4 and 5 – 'Historically, prior to the acceptance of the LAL method as comparable to the rabbit pyrogen test it replaced, the comprehensive studies were performed to assure that the LAL method could provide equivalent (or better) product quality decisions.'

> **Comment:** Comprehensive comparative studies were performed to assure that the LAL method could provide equivalent (or better) product quality decisions **rather than results**. This distinction may be useful in differentiating the use of LAL and

charles river

recombinantly derived reagents and help explain why recombinant reagents are considered alternative.

Regarding:

Page 4, **Comparability**, Line 2 – 'Any scientifically justified protocol designed specifically to compare assayable levels of autochthonous endotoxins from manufacturing sources....'

Comment: Further explanation of the word 'autochthonous' and how it relates to the Bacterial Endotoxin Test in terms of the naturally occurring endotoxins recovered from production relevant sources (e.g. within a water system) being tested is necessary. An explanation of how to assay the sample and identify organisms to check for autochthonous endotoxins is required.

Regarding:

Page 4, **Comparability**, Line 2 – 'Any scientifically justified protocol designed specifically to compare assayable levels of autochthonous endotoxins from manufacturing sources....'

and

Page 4, **Comparability**, Lines 14 to 16 – '...the test for comparability may be conducted by using a diluent during sample preparation (reconstitution and/or dilution of product) that consists of a phylogenetically diverse population of endotoxins that could be found in product or the manufacturing process'

Comment: If autochthonous is used to specifically address the organisms or population of organisms found in a particular place or from a particular source, understanding the species diversity present from that source is critical. It can be suggested that a thorough understanding of the microbial populations present in the sample can be achieved through next generation sequencing (NGS) technologies. These technologies are culture independent and can detect nucleic acids from live or dead cells that can contribute to the phylogenetically diverse population of endotoxins in the process. Many microorganisms may not grow well, or at all on agar plates, therefore a culture independent metagenomics application is needed to understand the complete microbiome. Metagenomics utilizes NGS to investigate microbial DNA or RNA obtained directly from environmental samples without cultivation and without prior knowledge of the constituent communities, and to generate a taxonomic profile of the microbial community.

Sincerely,

Jason J. O'Hare

Jason J. O'Hare, MSc.
Director Quality Assurance GLBL Operations, Regulatory Compliance
Microbial Solutions | Charles River

charles river

23rd November 2020

To Whom It May Concern

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Sincerely,

Jason J. O'Hare

Jason J. O'Hare, MSc.
Director Quality Assurance GLBL Operations, Regulatory Compliance
Microbial Solutions | Charles River

From:	Leslie Furr
To:	PF Comments
Subject:	FW: US Pharmacopoeia -- to enable use of recombinant Factor C (rFC)
Date:	Wednesday, November 25, 2020 12:55:19 PM

From: Church, George <gc@hms.harvard.edu>
Sent: Wednesday, November 25, 2020 7:21 AM
To: Leslie Furr <leslie.furr@USP.org>
Cc: HMS-church_lab_admin <church_lab_admin@hms.harvard.edu>; Heather Sparks <heather@reviverestore.org>; Ryan Phelan <ryan@reviverestore.org>
Subject: US Pharmacopoeia -- to enable use of recombinant Factor C (rFC)

November 25, 2020

Ms. Leslie Furr
Associate Scientific Liaison
The United States Pharmacopeial Convention, Inc.
12601 Twinbrook Pkwy.
Rockville, MD 20852
leslie.furr@usp.org

Subject: <1085.1> Use of Recombinant Reagents in the Bacterial Endotoxins Test — Pharmacopeial Forum PF 46(5)

Dear Ms. Furr,

As Professor of Genetics at Harvard Medical School (and HST at MIT) and head of Synthetic Biology at the Wyss Institute, I oversee the accelerated evolution of polymers and genomes to create new technologies with applications in regenerative medicine and bio-production of chemicals. I've co-founded several pharmaceutical companies, which, of course, screen for endotoxins. This letter is in response to uspnf.com/notices/bacterial-endotoxin-test-gen-announcement-20200828 request for comments.

It is critical to recognize that recombinant Factor C represents an innovative technological advance within the Bacterial Endotoxins Test using the clotting cascade found in *Limulus* Amoebocyte Lysate (LAL). Significant comparative literature continues to affirm that rFC is equivalent to or better than LAL for the detection of endotoxins and has been adopted in many other countries. Use of rFC should be an option. (It need not be a requirement).

The recombinant Factor C protein is the clone of the horseshoe crab's naturally produced Factor C, the bacterial endotoxins biosensor. Based on fundamental modern recombinant biotechnology principles, rFC is equivalent to the Factor C found in LAL. Because the manufacture of rFC can be standardized to a single genome sequence used to encode and therefore produce it, rFC can be made more consistently and therefore perform more consistently.

Compare this to LAL. The amebocyte blood is taken from living animals, which vary individually and per their environments, and the lysate preparation also varies among manufacturers. It is well known that this leads to differences in reactivity patterns across all compendial methods.

Unlike rFC which is composed entirely of a cloned Factor C, LAL also contains Factor B and Factor G. Factor G is a confounding factor in that it reacts with glucan. LAL can synergistically overpredict BET results in the presence of glucan, which is a very likely explanation for publicly-claimed disparities in rFC vs. LAL data for samples containing uncharacterized beta glucans.

Unfortunately, the proposed chapter <1085.1> only increases barriers to recombinant Factor C (rFC) implementation, potentially delays approval of new drug products, and discourages drug manufactures from utilizing rFC for BET testing.

I urge the US Pharmacopoeia to re-evaluate the proposed guidance to remove additional regulatory burdens to the use of rFC, which is a particularly urgent issue given the ongoing pandemic.

Sincerely,

--George

George Church, George Church, Professor of Genetics , Harvard Medical School, MIT,
Blavatnik Inst, Wyss Inst, Broad Inst, Regenesis Inst, SIAT Inst, PersonalGenomes.org
NRB room 238, 77 Avenue Louis Pasteur, Boston MA 02115
Web: arep.med.harvard.edu/gmc Advisory roles: v.ht/PHNc
All appointments via church_lab_admin@hms.harvard.edu

Leslie Furr

From: johannes.oberdoerfer@boehringer-ingelheim.com
Sent: Wednesday, November 25, 2020 3:57 AM
To: Leslie Furr
Subject: Comments on USP Chapter 1085.1 recombinant Factor C
Attachments: Response Letter USP Chapter 1085.1.docx

Dear Ms. Furr,

Attached please find my comments on the USP chapter 1085.1

Please note that this is my personal opinion and thus does not represent the official opinion of Boehringer Ingelheim or any of its affiliates.

Kind regards,
Johannes Oberdörfer

https://boehringer.sharepoint.com/sites/z365globaldigitalbiopharmateam/SitePages/Do-you-already-know-our-Digital-Ambassador-Program-.aspx

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Ms. Leslie Furr
Associate Scientific Liaison
The United States Pharmacopeial Convention, Inc.
12601 Twinbrook Pkwy.
Rockville, MD 20852 leslie.furr@usp.org

General Chapter <1085.1> Use of Recombinant Reagents in the Bacterial Endotoxins Test—Photometric and Fluorometric Methods Using Recombinantly Derived Reagents

Dear Ms. Furr:

I are writing in response to the draft Guidance posted 01-Sep-2020 for a new General Chapter <1085.1>, Use of Recombinant Reagents in Bacterial Endotoxins Test.

As you may know, our company has been evaluating the recombinant factor C (rFC) as an equivalent alternative to the biologically sourced Limulus amebocyte lysate (LAL) assay. I believe that rFC is equivalent and comparable to LAL. Our company has extensively tested (process water, drug components and intermediate products). I have found that rFC is equivalent to LAL in detecting bacterial endotoxins in injectable pharmaceuticals and is advantageous for us and for other manufacturers to use in ensuring the safety of our products.

Thus I were dismayed, first, when you abandoned progress toward revising the compendial chapter <85>, and second, instead of following the EMQD in creating a stand-alone chapter to direct the use of rFC (with the ultimate goal of harmonizing both with Ph.Eur. 2.6.32 and <85>), you instead created a guidance chapter that creates more, not fewer, barriers to adopting rFC than if you had left the status quo unchanged.

Unfortunately, the USP action adds unnecessary delay to incorporating rFC in a USP General Method. I hope that USP will engage all stakeholders equally and transparently to create solutions to bridge this gap in timing. I would much prefer that you move directly to a General Chapter similar to the European Pharmacopeia Ph.Eur. 2.6.32, either by amending <85> or creating a new sub-1000 chapter, e.g. <86>. But if you are determined to move forward with <1085.1>, I feel obliged to point out significant problems with the proposed chapter that must be resolved before it reaches its final form.

Most importantly, the proposed comparability guidance goes beyond FDA guidelines and published evidence for comparability and equivalence of the assays. Indeed, the additional work you are now requiring emerges not from weaknesses in rFC, but from the variability of LAL due to its biological origins and multiple manufacturers, and because LAL is subject to a range of artifacts, not least the enhancement of positive signals by the presence of glucans. Indeed, one could

conclude from your document that it is LAL that needs to be proven against rFC's high sensitivity, reproducibility simplicity, and elimination of false positives. In short, you are asking for rFC to achieve comparability with a more complex, less reproducible assay that is subject to elusive false positives. Why?

Thus, I request that you do the following:

· Remove from the proposed chapter <1085.1> occurrences of additional validation proof beyond what is already stated in 2012 FDA Guideline and <1225>, specifically the proposed comparability guidance.

· Remove from the proposed chapter <1085.1> the PTC on supplier quality as that is out of scope of public health standards

· Establish in short order a sub <1000> standalone chapter similar to Ph.Eur. 2.6.32, that can ultimately be merged into <85>, and which, importantly recognizes the scientific validity and comparability of rFC as does the EMQD.

I look forward to engaging with you further on this issue and will reach out to discuss how these matters may be moved forward in discussions with you.

Please note that this is my personal opinion and thus does not represent the official opinion of Boehringer Ingelheim or any of its affiliates.

Sincerely yours,

Johannes Oberdörfer

Lead Scientist Rapid Microbiological Methods at Boehringer Ingelheim

30 November 2020

To:
Leslie Furr
Associate Scientific Liaison
United States Pharmacopeia
12601 Twinbrook Pkwy
Rockville, MD 20852
Phone: 240-221-2022
leslie.furr@usp.org

From:
Veronika Wills
Manager, Technical Services
Associates of Cape Cod, Inc.
124 Bernard E. Saint Jean Drive
E. Falmouth, MA 02536
Phone: 508-444-1415
vwills@acciusa.com

Re: Comments to USP PF Online Proposal for <1085.1> Use of Recombinant Reagents in the Bacterial Endotoxins Test – Photometric and Fluorometric Methods using Recombinantly Derived Reagents (Correspondence Number – C273155)

To Whom It May Concern,

Associates of Cape Cod, Inc. (ACC) reviewed the proposal for the proposed chapter <1085.1> **Use of Recombinant Reagents in the Bacterial Endotoxins Test – Photometric and Fluorometric Methods using Recombinantly Derived Reagents.** Our detailed comments can be found in Table 1 of this letter.

We would like to offer a general comment concerning the Comparability studies: We at ACC understand the need to perform a scientifically justified protocol designed to compare the assayable levels of autochthonous endotoxins, however, additional questions remain. Given that it is difficult for a GMP drug or medical device manufacturer to obtain compendial samples containing assayable levels of autochthonous endotoxins in order to demonstrate comparability to compendial tests (e.g. LAL), we believe many end users will be looking for further guidance on alternatives to such samples. Page 4/6 describes that "*If manufactured drug product containing suitable levels of endotoxins activity is not available, the test for comparability may be conducted by using a diluent during sample preparation (reconstitution and/or dilution of product) that consists of a phylogenetically diverse population of endotoxins that could be found in product or the manufacturing process. Historically, the source of endotoxins entering manufacturing processes has most often been aquatic Gram-negative bacteria colonizing water systems (21-22). Thus, an appropriate analyte may be water taken from the upstream water for injection (WFI)*

purification stream after the carbon filters, for example, or in some cases deionized water may be used." Can this be further specified in the chapter as this is likely to become the focal point of the comparability evaluation? For example, can the expectation be specifically defined for:

-What type of process water to use: post-carbon filtration or deionized water?
-What should be the assayable levels of endotoxin in such water samples?
-What assayable levels of endotoxin should be present in the compendial sample following the preparation of the sample with the process water?
-Can other drug samples containing assayable levels of autochthonous endotoxin be also used in the comparability in absence of compendial samples? If so, what samples specifically?

The second general comment that we would like to point out is the suggestion to submit a Drug Master File with the regional authority. Recombinant reagents for BET are not drugs nor are they FDA-licensed products like LAL products therefore there is no requirement for a Drug Master File to be filed with the appropriate regional authority. There is no clarification provided as to why manufacturers should consider such a filing. Our suggestion is to replace this statement with the following: "It is required that the recombinant reagents are shown to be equivalent to LAL-based reagents. It is required for the end user of the recombinant reagents to demonstrate its suitability for the intended use as well as equivalence of the recombinant to LAL reagents of in their specific product(s). It is required that the data to support this in any filing are always available where endotoxin levels are established using recombinant products. Similarly, manufacturers of recombinant reagents shall have data demonstrating analytical performance, suitability and equivalency on file and available for review upon audit." This alternate statement reiterates in a closing statement that the importance of data gathering which together with a well-defined protocol shall be used when validating the use of the recombinant reagents at alternatives to LAL.

Please do not hesitate to contact me for any clarification on our feedback. Thank you for your consideration.

Yours respectfully,

Veronika Wills

DocuSigned by:
Veronika Wills
Signer Name: Veronika Wills
Signing Reason: I am the author of this document
Signing Time: 30-Nov-2020 | 2:15:25 PM EST
2A94A5CC800646DE873B292E68FD19F8

Table 1: Detailed feedback organized by page and section

Page no./Section	Current Text	Proposed Change	Justification
1/6 Background	*As in many innate immune responses, the hemolymph clotting process in the horseshoe crab includes a cascading series of zymogen proteases (1,3). In the horseshoe crab and in the in vitro lysate reaction, endotoxins bind to and convert Factor C to its active form, which subsequently activates Factor B, which in turn activates the proclotting enzyme. The activated proclotting enzyme then cleaves the targeted clotting protein, coagulogen, resulting in increased turbidity (kinetic turbidimetric assay) and, ultimately, a clot of the lysate sample mixture (gel clot test). The chromogenic assay is similar to the turbidimetric assay but, rather than cleaving coagulogen, the proclotting enzyme cleaves a chromophore from a colorless substrate, resulting in an increase in color intensity of the reaction mixture. The extent of turbidity or color is proportional to the level of endotoxins activity in the test solution that binds to Factor C, the first zymogen in the cascade.*	As in many innate immune responses, the hemolymph clotting process in the horseshoe crab includes a cascading series of zymogen proteases (1,3). In the horseshoe crab and in the in vitro lysate reaction, Factor C bind to endotoxins and are converted to its active form, which subsequently activates Factor B, which in turn converts the proclotting enzyme to clotting enzyme. The clotting enzyme then cleaves the targeted clotting protein, coagulogen, resulting in increased turbidity kinetic turbidimetric assay) and, ultimately, a clot of the lysate sample mixture (gel clot test). The chromogenic assay is similar to the turbidimetric assay but, rather than cleaving coagulogen, the clotting enzyme cleaves a chromophore from a colorless substrate, resulting in an increase in color intensity of the reaction mixture. The extent of turbidity or color is proportional to the level of endotoxins activity in the test solution that binds to Factor C, the first zymogen in the cascade.	Factor C binds to endotoxins as Factor C has the enzymatic activity. Proclotting enzyme is the zymogen of clotting enzyme.
3/6, Figure 3	*Yellow Color (endpoint and kinetic chromogenic)*	Yellow Color (kinetic chromogenic)	The rCR, to our, knowledge, are for kinetic assays only b
3/6	*Current literature suggests that the following may affect the sensitivity and/or specificity of the recombinant reagents and should be considered when choosing reagent suppliers (see below) and preparing comparability protocols: Choice of species of the horseshoe crab as the source of the recombinant reagents (11); Optimal number of cascade zymogen proteases in the recombinant reagent formulation (13,14); The type and control of cell lines used as the expression system (10 - 11); Identification and impact of any post-translational modification of the recombinant zymogen proteases (10-11, 15-16); The final, proprietary reagent formulation that assures consistent functionality and stability of the chosen recombinant*	Current literature suggests that there are many factors that may affect the sensitivity and/or specificity of the recombinant reagents (10,11,13,14,15,16) thus the emphasis on validation as alternate methods.	This paragraph may be shortened to avoid confusion of the end user. The differences in sensitivity and/or specificity of the recombination reagents are to be validated as described above in the document.

	components selected by the reagent manufacturer (10).		
Table 1	*Understanding reagent process development with respect to factors that may affect the sensitivity or specificity of the alternative test Documentation and assurance that the following do not affect the sensitivity O(specificity of the reagent and that changes to these elements are properly documented and controlled: Optimal choice of species of the horseshoe crab as the source of the recombinant proteins Optimal number of cascade zymogens present in the formulation Type and control (master/working cell banks) of the lines used as the expression system Identification and verification of any post-translational modifications Reagent formulation.*	Remove the entire row of the table.	This plays a role during the preliminary studies to determine if a change to a recombinant method is feasible (as documented on page 3/6) and full comparability study. To ensure continued quality, these aspects are covered in Table 1, second row under: "Materials and supplier management including certification of the raw materials and the consistency of product analytical performance; ……. Change management including notification to the user of any changes in formulation or manufacturing that may affect the test result."
5/6	*Though not required, manufacturers of recombinant endotoxin reagents should consider the submission of a drug master file of the equivalent depending on the regional authority.*	Replace with: "It is required that the recombinant reagents are shown to be equivalent to LAL-based reagents. It is required for the end user of the recombinant reagents to demonstrate its suitability for the intended use as well as equivalence of the recombinant to LAL reagents of in their specific product(s). It is required that the data to support this in any filing are always available where endotoxin levels are established using recombinant products. Similarly, manufacturers of recombinant reagents shall have data demonstrating analytical performance, suitability and equivalency on file and available for review upon audit."	Recombinant reagents for BET are not drugs nor are they FDA-licensed products like LAL products therefore there is no requirement for a Drug Master File to be filed with the appropriate regional authority. There is no clarification provided as to why manufacturers should consider such a filing.
References	*ASTM E2935-17*	ASTM E2935-17	ASTM E2935-17 was superseded by E2935-20
Figure 1	Glucan	β-glucan	
Figure 2	rFC Cascade	rFC fluorogenic reaction	

NJPQCA

New Jersey Pharmaceutical Quality Control Association

27 November 2020

Ms. Leslie Furr
Associate Scientific Liaison
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Subject: <1085.1> Use of Recombinant Reagents in the Bacterial Endotoxins Test—Photometric and Fluorometric Methods Using Recombinantly Derived Reagents
Reference: Pharmacopeial Forum 46(5) – Comment Deadline: 30-Nov-2020

Dear Ms. Furr,

NJPQCA is an organization, established in 1954, whose membership encompasses pharmaceutical industry professionals from across the United States with a mission to encourage and stimulate dialogue among Quality Assurance/Control and Regulatory Compliance professionals by providing forums and networking opportunities for the exchange of views on technical topics and regulatory issues relevant to the pharmaceutical industry. NJPQCA's Compendial Discussion Group is comprised of about 50 professionals responsible for monitoring compendial changes for approximately 20 pharmaceutical companies, most of which are multi-national.

NJPQCA would like to provide the following comments and recommendations regarding the new general chapter proposal <1085.1>, *Use of Recombinant Reagents in the Bacterial Endotoxins Test—Photometric and Fluorometric Methods Using Recombinantly Derived Reagents,* in PF 46(5):

1. Members within NJPQCA value a scientific approach to expanding the use of recombinant Factor C for the detection of Bacterial Endotoxins. However, The proposed comparability testing per draft <1085.1> requires an undue burden of proof which is not consistent with <1225> and equivalent global pharmacopoeia chapters, or the FDA Guidance: Analytical-Procedures-and-Methods-Validation-for-Drugs-and-Biologics.

   Additionally, references to <1223> are not applicable as the Bacterial Endotoxin Testing Chapter <85> resides under Biological Tests and Assays, and not Microbiological Tests and Assays in the USP. Therefore, <1225> is the guiding principle which is affirmed in the FDA Guidance.

2. NJPQCA holds that as written, the proposed chapter <1085.1> increases barriers to rFC implementation, potentially delays approval of new drug products, and practically prevents the conversion of registered LAL methods to rFC methods. In contrast, we propose the following recommendations:

   - The confirmation testing for a chosen rFC as a suitable alternative reagent for use per General Chapter <85>, Bacterial Endotoxins Test, can be outlined through reagent suitability tests within the USP. Such processes that demonstrate the reagent meets the requirements for its intended application would be verified by the user.
   - By building the requirements for using rFC into Chapter <85> as national text, the USP would not need to revisit PDG harmonization, and companies would be spared the lengthy re-submission processes that delay regulatory approvals.

3. The section on Supplier Quality is redundant to existing regulatory guidelines through established regulatory agencies (e.g., EMA, FDA, ICH) that provide the 'cGMPs' for the oversight of suppliers to the pharmaceutical industry. Quality system content is under the purview of a firm per cGMP law, therefore it is out of place to specify quality system practices in a pharmacopoeia method guidance chapter. This section should be removed.

4. In contrast to the statement within the draft <1085.1> that: "*compendial articles containing assayable levels of endotoxins activity from autochthonous endotoxins are not available or have not been published in the public domain*," we offer the following list of published, peer-reviewed articles that provide head-to-head rFC:LAL comparisons of environmental endotoxin data:

Author	**Title / Journal, Year**
Alwis, K. U., and D. K. Milton	Recombinant factor C assay for measuring endotoxin in house dust: comparison with LAL, and (1–3)- -Dglucans / Am. J. Ind. Med., 2006.
Thorne et al	Evaluation of the Limulus Amebocyte Lysate and Recombinant Factor C Assays for Assessment of Airborne Endotoxin / Appl Environ Micro, 2010.
McKenzie et al	Evaluation of lot-to-lot repeatability and effect of assay media choice in the recombinant Factor C assay / J Environ Monit, 2011.
Chen and Mozier	Comparison of Limulus amebocyte lysate test methods for endotoxin measurement in protein solutions / J Pharm Biomed Anal, 2013.
Schwarz et al	Residual Endotoxin Contaminations in Recombinant Proteins Are Sufficient to Activate Human CD1c+ Dendritic Cells / PLoS ONE, 2014.
Bolden and Smith	Application of Recombinant Factor C Reagent for the Detection of Bacterial Endotoxins in Pharmaceutical Products / J Pharm Sci Technol, 2017.
Kikuchi et al	Collaborative Study on the Bacterial Endotoxins Test Using Recombinant Factor C-based Procedure for Detection of Lipopolysaccharides Part 1 / Pharm & Med Device reg Science, 2017.
Marius et al	Comparison of LAL and recombinant Factor C assays for endotoxin detection in four human vaccines with complex matrices / J Pharm Sci Technol, 2020.
Bolden	Application of Recombinant Factor C Reagent for the Detection of Bacterial Endotoxins in Pharmaceutical Products and Comparability to LAL

5. Industry associations have evaluated the acceptability of rFC reagent use. In August 2020, the Parenteral Drug Association (PDA) published a review by a panel of industry, regulatory and compendia bacterial endotoxins subject matter experts of the existing literature and concluded that rFC is comparable to LAL.

In summary, NJPQCA believes the USP should engage all significant stakeholders and drive to a General Test within Chapter <85> that includes as an option the use of rFC reagent, as originally proposed in PF 45(5). We encourage the USP to provide flexibility in the guidance chapter <1085.1> and within any reagent suitability tests adopted within the USP for rFC.

NJPQCA appreciates the opportunity to comment on the new chapter proposal <1085.1>, and we hope that our comments and recommendations are taken into consideration.

Sincerely

Hantz Tattegrain
Chair, NJPQCA Compendial Discussion Group

PhysiciansCommittee
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November 30, 2020

Ms. Leslie Furr
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RE: ⟨1085.1⟩ Use of Recombinant Reagents in the Bacterial Endotoxins Test - Photometric and Fluorometric Methods Using Recombinantly Derived Reagents - PF 46(5)

Submitted via online.usppf.com/

Dear Ms. Furr:

The Physicians Committee for Responsible Medicine is a nationwide nonprofit representing 175,000 members working for effective, efficient and ethical medical research and product testing. We collaborate with federal agencies and the regulated industry to improve reliability, accuracy, and supply chain stability in pharmaceutical safety testing. We write in support of the USP moving to recognize recombinant Factor C (rFC) reagents as equivalent to horseshoe crab blood-sourced zymogen protease preparations utilized in the existing compendial method of bacterial endotoxins testing (BET). **Specifically, we ask that the USP amend Bacterial Endotoxins Testing chapter <85> to include rFC as a compendial method. Alternatively, the text proposed in <1085.1> should be modified as described below, and drafted as a sub <1000> chapter.**

Consistent with the USP's description of the benefits to use of rFC outlined in PF 46(5)[1], we find three crucial benefits to using rFC over *Limulus* amoebocyte lysate (LAL) and *Tachypleus* amoebocyte lysate (TAL) assays:

- "The elimination of the use of animals as a reagent source
- The absence of the glucan pathway that can result in a non-endotoxin-specific enhancement of the lysate test results

[1] USP, <1085.1> Use of Recombinant Reagents in the Bacterial Endotoxins Test – Photometric and Fluorometric Methods Using Recombinantly Derived Reagents

- The potential for a more consistent and stable supply of reagents not subject to market and environmental pressures"

These benefits, combined with scientific evidence demonstrating rFC is at minimum scientifically equivalent to horseshoe crab tests in detecting endotoxin, justify the need for swift and responsible action by the USP to remove hurdles to use of rFC.

Earlier this year, the European Pharmacopoeia introduced a new chapter on rFC, Eur. Ph. 2.6.32,[2] in response to the available data[3] and the need for a more reliable analytical reagent. This chapter was published in July of 2020 and will be effective January 1, 2021. A similar effort was expected to be adopted by the USP, however, in May the drafted revision to USP <85> was rescinded and a guidance chapter <1085.1> was instead proposed. Rather than removing barriers to use of rFC, as proposed, under the new guidance in <1085.1>, companies will have to provide additional evaluation data to use rFC that goes beyond what is currently required by USP <1225> and by the FDA.[4]

The rFC Assay Represents an Alternative Reagent Source, not an Alternative Method

While defined in the proposed text as an "alternative method" for the purposes of USP, the rFC assay should not be categorized as an alternative method because it does not introduce a novel assay principle. Rather, it should be considered an "alternative procedure" as it utilizes the same in vitro Bacterial Endotoxins Test principle as the LAL assay. The early development of the BET assay platform and its path of acceptance in lieu of the rabbit pyrogen test is not a reasonable analogy to the acceptance of the alternative sourcing of protease reagents needed to carry out the BET assay, now that BET has been fully established as compendial. Rather, an appropriate analogy for the inclusion of rFC in <85> would be the introduction of the chromogenic substrate or other technological advances improving properties of the components of the activation cascade. The only significant difference when moving from LAL to rFC is that the initial zymogen protease in the LAL cascade, Factor C, is made via recombinant protein production rather than derived from horseshoe crab blood. The recombinant enzyme is stable and consistent,[5] and recombinant protein production is a technique that has existed for nearly forty

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[2] Recombinant factor C: new Ph. Eur. chapter available as of 1 July 2020. https://www.edqm.eu/en/news/recombinant-factor-c-new-ph-eur-chapter-available-1-july-2020

[3] 1. Ding, J. L.; Ho, B., A new era in pyrogen testing. Trends in Biotechnology **2001**, 19 (8), 277-281. **2.** Bolden, J. S.; Smith, K. R., Application of recombinant Factor C reagent for the detection of bacterial endotoxins in pharmaceutical products. PDA Journal of Pharmaceutical Science and Technology **2017**, 71(5), 405-412. **3.** Piehler, M. et al. Comparison of LAL and rFC Assays-Participation in a Proficiency Test Program between 2014 and 2019. Microorganisms **2020**, 8**,** 418.**4.** Bolden J, Knutsen C, Levin J, et al. Currently Available Recombinant Alternatives to Horseshoe Crab Blood Lysates: Are They Comparable for the Detection of Environmental Bacterial Endotoxins? A Review. PDA Journal of Pharmaceutical Science and Technology. **2020**, 74(5), 602-611.

[4] FDA Guidance for Industry, Pyrogen and Endotoxins Testing: Questions and Answers, **2012**

[5] Mizumura H., et al. Genetic engineering approach to develop next-generation reagents for endotoxin quantification. Innate Immunology. 2017, 23(2), 136-146.

years to generate bioactive enzymes in a controlled and consistent environment. Methods based on rFC, therefore, should not be defined as alternative methods subject to additional validation requirements beyond what is necessary to validate other new LAL formulations and testing platforms.

rFC is Scientifically Advantageous to LAL because rFC is Endotoxin-Specific

The most significant improvement shared by rFC and other recombinant assay platforms is the elimination of the nonspecific activation of the clotting cascade through Factor G, which is activated by lipopolysaccharides and similar non-pyrogenic cell wall components common to both bacteria and fungi. This nonspecific activation pathway common to all *Limulus* and *Tachypleus* amoebocyte lysates leads to falsely elevated endotoxin results in the presence of impure samples containing nonpyrogenic materials such as beta glucan, which are only partially blocked by buffers designed to reduce this effect. Notably, this nonspecific activation can lead to both false-positives in the absence of endotoxin, as well as falsely enhanced quantification through synergistic activation between endotoxin and beta-glucans or other interfering compounds.

Recent literature strongly supports both the arguments that rFC is equivalent to LAL for the detection of endotoxins and that LAL is over-predictive due to nonspecific activation of the protease cascade. For example, the Japanese comparability study by Kikuchi and colleagues[6] consistently demonstrates that LAL and rFC are statistically indistinguishable when detecting naturally occurring endotoxins in pharmaceutical production, irrespective of whether steps were taken to eliminate the nonspecific activation pathway in LAL. However, when unpurified water was used, elevated measurements are observed for the unblocked LAL assay for every sample tested when compared to the LAL assays where nonspecific interference was mitigated. Accordingly, the most rational explanation for observed differences between assays in the unpurified water samples is nonspecific interference. This emphasizes the importance of characterized samples, rather than unpurified water, for analysis of comparability between LAL and rFC results.

Given the variety of nonspecific interferences that can lead to falsely elevated LAL results,[7] it is not scientifically feasible to evaluate rFC accuracy using autochthonous or indigenous samples. For this purpose, only samples that are prepared without such interference are appropriate. Reference Standard Endotoxin (RSE) and Control Standard Endotoxin (CSE) are used to

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[6] Kikuchi, Y. et al., Collaborative Study on the Bacterial Endotoxins Test Using Recombinant Factor C-based Procedure for Detection of Lipopolysaccharides. Pharmaceutical and Medical Device Regulatory Science **2017**, 48, 252-260.

[7] **1.** Roslansky P.F., Novitsky T.J. Sensitivity of Limulus amebocyte lysate (LAL) to LAL-reactive glucans. Journal of Clinical Microbiology **1991**, 29(11), 2477-83. **2.** Cooper, J.F., Weary, M.E., Jordan, F.T. The impact of non-endotoxin LAL-reactive materials on Limulus amebocyte lysate analyses, PDA Journal of Pharmaceutical Science and Technology **1997**, 51 (1): 2-6. **3.** Pearson F.C., et al. Characterization of Limulus amoebocyte lysate-reactive material from hollow-fiber dialyzers. Applied Environmental Microbiology **1984**, 48(6), 1189-1196.

calibrate all commercially available LAL assays, although there is expected to be some minor variance in response between calibration controls and measured samples. Given the inherent dependence of all bacterial endotoxin quantification on these standards, there is no reason that these standards are not adequate for evaluating the relative quantification of LAL and rFC assays. However, as written, the proposed text of chapter <1085.1> provides no standardized guidance for a reliable means to eliminate nonspecific interference from beta-glucans or any other LAL-reactive microbial sugars likely to be present in pre-filtration water samples.

Relying on a Single Source for BET During a Global Pandemic is an Unnecessary Risk

Horseshoe crab sourced lysates are a marine natural product, making them inherently variable and unpredictable. The process for producing LAL, which is derived from a complex bleeding operation requiring harvesting horseshoe crabs from the Atlantic coast, is inconsistent from lot to lot due to natural biological variations.[8] Because horseshoe crab populations are threatened, it will also be difficult to quickly increase production when needed. By contrast, recombinant protein production has been used for nearly forty years to generate bioactive enzymes in a controlled and consistent environment. Sourcing active enzymes from laboratory cultures has the advantage of being scalable and alleviates supply chain issues inherent to global dependence on a marine species for patient safety.

Conservation groups have sought to bring attention to the vulnerability of the last available horseshoe crab species and the complex ecosystem it supports since the 1990s. Unfortunately, as both biomedical and bait harvests have increased in recent years, the many species reliant on this irreplaceable creature's predictable breeding habits are showing no signs of recovery. Annual harvest rates reported by the Atlantic States Fisheries Management Commission show that the average increase in biomedical bleeding harvests, year-over-year since 2016, has been more than 90,000 horseshoe crabs.[9] As both harvests and associated mortality have continued to steadily increase leading to record high levels prior to the SARS-CoV-2 pandemic, the risk of relying on a single keystone species to ensure patient safety is unacceptable.

Necessary Modifications to Proposed Chapter <1085.1>

The proposed text for chapter <1085.1> needs extensive modification to reflect an appropriate evaluation of both compendial and recombinant bacterial endotoxins tests. Ideally, the text of chapter <85> should be amended to promote the use of recombinant BET methods such as rFC, or a sub-1000 chapter drafted to describe the evaluation of recombinant BET methods, limited to product-specific validation via <1225> as is currently the expectation for compendial methods from different manufacturers. Because rFC assays are not more variable than the different LAL preparations available, additional requirements are unnecessary and without precedent. These have never been applied to previous modernized implementations of LAL clotting cascade-based

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[8] Milton D.K., et al. Environmental endotoxin measurement: interference and sources of variation in the Limulus assay of house dust. American Industrial Hygeine Association Journal.1997, 58(12), 861-7.
[9] Review of the Interstate Fishery Management Plan - Horseshoe Crab (Limulus polyphemus) 2019 Fishing Year. http://www.asmfc.org/uploads/file/5f99c5a32019HorseshoeCrabFMP_review.pdf

assays such as introduction of the chromogenic endpoint. A suitable compendial reference chapter for recombinant assays should reflect the following:

- Recognize the complexity and variability of various LAL manufacturers' preparations precludes the use of LAL assays to determine a "true" endotoxin value
- There is no necessity for alternative method validation as described in the section entitled "Comparability"
    - The use of RSE and/or CSE is sufficient for validation of rFC comparability to LAL via the relevant requirements described in <1225>.
    - Validation of recombinant enzyme assays via <1223> is inappropriate, as Bacterial Endotoxins Tests are defined as Biological Tests and Assays, rather than Microbiological Tests and Assays.
- Information on Supplier Quality is not relevant in a compendial chapter for end users
    - Details including horseshoe crab species and proteases used for the formulation, the cloning system, and stability and functionality of the preparation are managed by the vendor and incorporated into the assay's specifications.
    - End users should implement normal quality control measures for rFC including selecting an assay with the appropriate limit of detection and confirming performance standards as necessary.

In conclusion, relevant literature demonstrates that just as LAL assays have been improved by introducing quantitative chromogenic substrates, rFC is an improved source of zymogen proteases for detecting endotoxins using the same assay principle used in LAL- and TAL-based detection systems. Nearly two decades of analyses have demonstrated that recombinant assays give equivalent results to traditional LAL and TAL assays in response to prepared bacterial lipopolysaccharides as well as naturally-occurring endotoxins. Additionally, rFC has the advantage of being endotoxin-specific, without the alternative pathways that can lead to falsely elevated results in LAL. And, finally, rFC eliminates the use of horseshoe crabs, resulting in healthier ecosystems and overcoming potential supply chain issues inherent in relying on a limited natural resource by using a scalable enzyme source.

For these reasons, we ask the USP to amend Bacterial Endotoxins Testing chapter <85> to include rFC as a compendial method. Alternatively, the text of chapter <1085.1> should be modified and included in a sub-<1000> chapter.

Thank you for reviewing our input. Please contact us with any questions; we welcome a discussion.

Sincerely,

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<1085.1> Use of recombinant reagent in the Bacterial Endotoxin Test

US-PF 46(5) Sep-Oct 2020 ***C273155***

- Comments submitted by: Vaccines

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- Date : November 30th, 2020

General Comments
Thank you for the opportunity to provide comments on this draft chapter. Please find below our comments.

Detailed Comments				
Document page / section	**Paragraph / line N°**	**Text commented**	**Comment / Rationale**	**Proposed change / suggested text**
All document	All document	All document	Thanks to let us the opportunity to comment this new guideline. We are a pharmaceutical company with significant experiences with current commercialized rFC reagents and we have already published some articles this year 2020 on that subject in peer review = in PDA journal that you cited in this new guideline (references number 19) with different biological matrices but also with different grade of pharmaceutical water in Journal Biologicals sept 2020 “Comparison of bacterial endotoxin testing methods in purified pharmaceutical water matrices” same authors and finally in A3P La Vague n°66 July 2020 “Vaccines & endotoxin – a challenging world”. We are in the process through our experiences to implement rFC for the future replacement of LAL testing when possible. It is quite confusing for a final user in pharmaceutical industry what is the exact strategy from USP about BET/rFC based method? We have followed the introduction in draft in chapter <85>, then his cancellation and now the introduction of this new guidelines which appears more as a barrier difficult to cross than a guide to help a future user. Finally, different paragraph in this guidelines (comparability, high requirement for the supplier of rFC or rCR, use of upstream water after carbon filters or deionized	Based on all the publication in peer review on rFC (more than 20), based on feedback by concrete experience of Analysts in Pharmaceutical companies, and based on feedback from all players in recombinant and non-recombinant BET testing (not only Charles River but also ACC, Lonza and bioMérieux) Given the existing publications that have already demonstrated the sensitivity but also the specificity of the rFC currently available from different suppliers, rethinking a guideline that helps a future user of rFC testing, even if still considered as an alternative method, in a simple and encouraging way.

Detailed Comments				
Document page / section	**Paragraph / line N°**	**Text commented**	**Comment / Rationale**	**Proposed change / suggested text**
			water) from our own interpretation seems coming from an internal study from Charles River CRL that is not published in a peer review. Moreover, Charles River has communicated a lot since this summer on its internal study via presentations in congress, linkedIn communication and Webinar against commercialized FC reagents and by frightening the future potential user.	
Page 1	background	*After figure 1. By design, recombinant reagents lack the alternative Factor G pathway where the presence of β-D-glucan can activate Factor G, which in turn can act as a non-endotoxin-specific activator of the proclotting enzyme*	Not only Beta D glucan could activate the LAL cascade but different LAL-RM (LAL-Reactive materials) like cellulosic residues, etc…the term more exact is LAL RM and not all LAL RM are currently known.	By the presence of LAL Reactive material (like 1,3 Beta D glucans, 1,4 Beta D glucans, Curdlans, Lamarin, Cellulose, fibers…) alternative Factor G pathway where the presence of reactive material such as β-D-glucan **but not limited to** can activate Factor G, which in turn can act as a non-endotoxin-specific activator of the proclotting enzyme.
Page 3	Validation of alternative method	*Chapter <1223>*	Since 2012, FDA asked for use of only chapter <1225> to validate rFC as alternative method. <1223> described microbiological method based on detection and enumeration of microorganisms and based on CFU. Very far away from BET tests based on LAL or rFC. Deletion of Validation of Alternative Microbiological Methods ⟨1223⟩ since this text is not applicable to endotoxin testing. This reference brings confusion.	Delete the microbiological alternative method requirement and <1223> and keep only <1225> as request from FDA. Delete part 3 comparability and used of autochthonous endotoxins. Specificity have been already demonstrated in different publication including two from Dr Kikuchi. We have internal data also with wild LPS showing specificity of rFC
Page 3	Validation of alternative method	*preparing comparability protocols:* • *Choice of species of the horseshoe crab as the source of the recombinant reagents (11)*	We are not in favor of comparability protocol and a comparability protocol couldn't take into account the choice of horseshoe crab, number of cascade zymogen, type and control cell lines	Delete 3 comparability

Detailed Comments				
Document page / section	**Paragraph / line N°**	**Text commented**	**Comment / Rationale**	**Proposed change / suggested text**
		• *Optimal number of cascade zymogen proteases in the recombinant reagent formulation (13–14)* • *The type and control of cell lines used as the expression system (10–11)* • *Identification and impact of any post-translational modification of the recombinant zymogen proteases (10–11, 15–16)* • *The final, proprietary reagent formulation that assures consistent functionality and stability of the chosen recombinant components selected by the reagent manufacturer (10)*	used and identification and impact of any post translational modification of the r zymogen proteases. This is research based only, not for a pharmaceutical industry analytical QC protocol execution.	
Page 2	Background	There are benefits to the adoption of recombinantly derived reagents: • The elimination of the use of animals as a reagent source • The absence of the glucan pathway that can result in a non-endotoxin-specific enhancement of the lysate test result • The potential for a more consistent and stable supply of reagent not subject to market and environmental pressures	Addition of benefits since missing and important and demonstrated by pharmaceutical companies	Addition: There are benefits to the adoption of recombinantly derived reagents: • The elimination of the use of animals as a reagent source • The absence of the glucan pathway that can result in a non-endotoxin-specific enhancement of the lysate test result • The potential for a more consistent and stable supply of reagent not subject to market and environmental pressures Robustness, reproducibility and stability of the reagent
Page 4	Validation of alternative	*In addition, unlike with conventional lysate reagents described in (85), the*	Why this requirement for a rFC? LAL is derived from an animal source that's	Delete this specific requirement the supplier

Detailed Comments				
Document page / section	**Paragraph / line N°**	**Text commented**	**Comment / Rationale**	**Proposed change / suggested text**
	method	*manufacture,….. Data from preliminary studies may need to be submitted to the regional regulatory authority as part of the method validation package.*	why it is FDA regulated, not rFC. Of course, pharmaceutical industry have experiences in management and control of critical reagents. But if this requirement for supplier of rFC are request then ask the same requirement for all safety reagent alterntive or not : for example, reagent used for alternative sterility test : ATP bioluminescence reagent, media used for BactAlert or Bactec detection, etc….and the future chapter <72> and <73>…	
Page 4	comparability	*Any scientifically justified protocol designed specifically to compare assayable levels of autochthonous endotoxins from manufacturing sources*	RSE, CSE and NOEs could be used. There are no autochthonous endotoxins from manufacturing sources standardized and well described! For example, for alternative sterility test we use ATCC strains (compare to RSE) and wild isolate from manufacturing (like NOEs even if not standardized). There is no validation of alternative sterility test based on real low contaminated samples…	Delete this requirement of comparability
Page 4	comparability	*Historically, prior to the acceptance of the LAL method as comparable to the rabbit pyrogen test it replaced, comprehensive studies were performed to assure that the LAL method could provide equivalent (or better*	This is claimed by Charles River. It's really not the same situation when in 40-60s only RPT existed and then replace in 70-80s by BET. Pyrogens in animals RPT and BET are completely different test. Here it's BET testing and replacement of a reagent by a recombinant one.	Delete the citation on RPT
Page 4	comparability	*Thus, an appropriate analyte may be water taken from the upstream water for injection (WFI) purification stream*	Again, this is claimed by Charles River. But unprocess, upstream water, sample after carbon filters, samples after	Delete the use of upstream purified water

Detailed Comments				
Document page / section	**Paragraph / line N°**	**Text commented**	**Comment / Rationale**	**Proposed change / suggested text**
		after the carbon filters, for example, or in some cases deionized water may be used. Because water used in the manufacture of parenteral products is a critical control point for endotoxins, it represents the types of endotoxins that may eventually contaminate the product.	deionized water are not good choice to compare LAL and rFC, because this kind of samples contains LAL Reactive Material…not only Glucans but cellulosic and fibers residues that activate positively the LAL reactions. This samples are not well characterized nor controlled in pharmaceutical industries. Only real final point of used purified samples contaminated by a biofilm could eventually used, but difficult to be used in real life with an artificial biofilm creation.	
Page 4	comparability	Given that the recombinant reagents have no Factor G pathway, the use of a glucan blocker for the lysate reagent is highly recommended. This will reduce any effects of glucans on the lysate that may alter the comparability test result.	It has been demonstrated the glucan blocker does not block 100% the Factor G pathway. In presence of glucan in the samples, there are synergistic effects with endotoxins thus altering the comparability test result. The LAL assay in that case will not give the true endotoxin content.	N/A
Page 5	comparability	It is suggested that comparability studies employ the USP Endotoxin RS for calibration curves and positive product control (PPC) in order to eliminate any effects that an unmatched combination of reagent lot–CSE lot may have on the test result.	For many years, the CSE from LAL suppliers has been used. CSE is calibrated against the RSE by the supplier (same potency). RSE stocks might not be enough for the worldwide use?	<u>Deletion of all sentence</u>
Page 5	Comparability	An example of acceptance criteria comparing measured endotoxins activity using recombinant reagents and naturally derived lysates might be the following: The measured activity of a sample containing endotoxins	In some examples, it has been demonstrated that recombinant reagent gives the true endotoxin content while in LAL it has been overestimated (presence of protease or glucans for example)	<u>Addition:</u> An example of acceptance criteria comparing measured endotoxins activity using recombinant reagents and naturally derived lysates might be the following: The measured activity of a sample containing

Detailed Comments				
Document page / section	**Paragraph / line N°**	**Text commented**	**Comment / Rationale**	**Proposed change / suggested text**
		using a recombinant reagent should fall within 50%–200% of the measured activity in the same sample tested using naturally occurring lysate as described in ⟨85⟩.		endotoxins using a recombinant reagent should fall within 50%–200% of the measured activity in the same sample tested using naturally occurring lysate as described in ⟨85⟩ if previously demonstrated appropriate.

November 30, 2020

Leslie Furr Specialist Microbiologist, NRCM
Associate Scientific Liaison
US Pharmacopeia
12601 Twinbrook Pkwy,
Rockville, MD 20852

RE: General Chapter <1085.1>, "Use of Recombinant Reagents in the Bacterial Endotoxins Test—Photometric and Fluorometric Methods Using Recombinantly Derived Reagents."

Dear Ms. Furr,

Please consider this correspondence, submitted on behalf of New Jersey Audubon and its 18,000 members, and National Wildlife Federation and its 6 million members and supporters, a response to the U.S. Pharmacopeia's draft Guidance posted in PF46(5) for a new General Chapter <1085.1>, "Use of Recombinant Reagents in the Bacterial Endotoxins Test—Photometric and Fluorometric Methods Using Recombinantly Derived Reagents."

For several reasons, we believe that USP is on the wrong track regarding its position on recombinant Factor C (rFC) and plans to move forward an information chapter, <1085.1> rather than including it in compendial chapter <85>. The latter would treat rFC as an equivalent to *Limulus* Amebocyte Lysate (LAL) and bring USP into alignment with its European counterpart, EDQM.

First, our review of the primary literature, in collaboration with experts in microbiology, suggests that sufficient data exists comparing rFC and LAL in head-to-head trials to treat them as equivalent. These have occurred across a range of diverse pharmaceutical products and manufacturing processes using "real-world" samples. For this reason, we think USP's stated requirement for additional validation for new and previously approved products is unwarranted and creates an undue burden beyond what is stated in FDA Guideline on Analytical Procedures and Methods Validation for Drugs and Biologics and <1225>.

Second, LAL is derived from a marine organism. This means LAL is subject to unpredictable and uncontrollable environmental factors that make batches produced by a single manufacturer, as well as among different manufacturers, more variable and thus less predictable than a reagent

manufactured using recombinant technology. USP does not require end users to compare or validate in head-to-head trials when they switch to different LAL suppliers.

Finally, USP's position on rFC means a reliance on a single source, LAL, for bacterial endotoxin testing reagents during a global pandemic. In this COVID-19 health crisis landscape, it is crucial that pharmaceutical manufacturers have the option to use rFC endotoxin test kits without implementing complicated and expensive validation and head-to-head comparisons with LAL. Failing to remove impediments by not revising Compendial Chapter <85> and advancing General Chapter <1085.1>, could hinder the rapid deployment of vaccines and therapeutics being developed by several pharmaceutical companies to combat COVID-19.

Although LAL producers claim that horseshoe crab blood is limitless and scaling up LAL production to meet demands associated with development and deployment of COVID-19-related prophylaxis, or future pandemic challenges, is erroneous. It neglects that Atlantic horseshoe crab populations have declined precipitously since the mid-1990s and have not recovered since the Atlantic States Marine Fisheries Commission imposed harvest restrictions in the early 2000s.

Assuming that horseshoe crab populations are sustainable and being managed as such, is short-sighted. In 2019 (i.e., pre COVID-19), biomedical harvest of horseshoe crabs increased nearly 50% compared to 2018 and mortality associated with blood extraction increased by 60%. That pressure on existing stocks of horseshoe crabs to meet increasing demand is unlikely, as LAL manufacturers have professed, is unrealistic and could result in a public health risk.

Relying exclusively on LAL for endotoxin testing represents a sole source scenario that hampers supply-chain capacity when fulfilling unanticipated demand is critical, e.g., COVID-19. Clearly, USP should acknowledge that multiple sources, not just multiple manufacturers dependent on the same sole source for critical quality reagents, is essential.

We urge the USP to stay the course and revise General Chapter <85> rather than move forward Chapter <1085.1> as this will be the most expeditious path forward to address public safety, reduce endotoxin reagent supply-chain challenges, decrease dependency on animal-derived biomedical products and significantly contribute to near-shore marine ecosystem viability. We welcome an opportunity to discuss any or all these issues with the USP now or in the future.

Sincerely,

David Mizrahi, Ph.D.
Vice-president, Research and Monitoring

Curtis Fisher
Executive Director
National Wildlife Federation
Northeast Regional Center

Dr. Leslie Furr
Associate Scientic Liaison
United States Pharmacopeial Convention, Inc.
12601 Twinbrook Parkway
Rockville, MD 20852

November 30, 2020

RE: USP PF 46 (5) ⟨1085.1⟩ Use of Recombinant Reagents in the Bacterial Endotoxins Test—Photometric and Fluorometric Methods Using Recombinantly Derived Reagents

Dear Dr. Leslie Furr,

Novo Nordisk appreciates USP's efforts to implement the general chapter and is pleased to provide comments on the proposed new chapter of USP as published in the *Pharmacopeial Forum*.

Novo Nordisk is a pioneer in biotechnology, a world leader in diabetes care and holds a leading position within hemostasis management, growth hormone therapy, and hormone therapy for women. Novo Nordisk manufactures and markets pharmaceutical products and services that make a significant difference to our patients, the medical profession, and society.

Our comments below note areas where we make recommendations or request further clarification.

High Priority Comments			
Section	**Current text**	**Suggested text**	**Rationale for Comments**
Briefing		Remove reference to <1223>	Test for bacterial endotoxins is not regarded as a microbiological method and thus <1223> do not apply.
Background	(kinetic turbidimetric assay)	(turbidimetric assay)	Both endpoint and kinetic assay exists

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Validation of alternative methods		Remove reference to <1223>	Test for bacterial endotoxins is not regarded as a microbiological method and thus <1223> do not apply.
Validation of alternative methods	Endotoxins from autochthonous manufacturing sources	Delete or rewrite	If the term "from autochthonous manufacturing sources" is used it must be further explained. Validation of an alternative method using endotoxins that are not characterized and standardized is not appropriate. If use of non-purified endotoxin is required further guidance must be given in order to ensure some kind of consistency. Please provide the scientific argument for using it in addition to RSE/CSE.
Validation of alternative methods	"…the user of these reagents to assure that…."	Align with requirements for other similar reagents	More strict requirements for recombinant reagents for BET than other reagents
Validation of alternative methods	"Data from preliminary studies may need to be submitted to the reginal regulatory …validation package"	Rewrite	For submission, it should be sufficient to provide the final validation data for documentation of validation activities.
Comparability	"…comprehensive data demonstrating comparability…"	Rewrite	Data are available. Please refer to PDA paper published earlier this year.

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Comparability	"…CSEs and RSEs may be used in addition…"	Rewrite	Difficult to perform comparability with an "autochthonous endotoxins sample" that may contain elements other than endotoxins that can activate the LAL cascade. Please provide the scientific argument for using it in addition to RSE/CSE.
Points to consider	Entire section	Remove	Procedure for ensuring supplier quality should not be included. It is not specific for recombinant reagents and it is not part of description for other similar reagents in USP. General GMP requirements should not be included in the chapter.

Thank you for the opportunity to provide comments. If you have any questions, please contact mr. Anders Thorn (adet@novonordisk.com) and Quality_Intelligence@novonordisk.com

On behalf of Novo Nordisk
Sincerely,

Susanne Herrguth
Quality Intelligence Manager
Novo Nordisk A/S

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From:	Leslie Furr
To:	PF Comments
Subject:	FW: Comments on 1085.1----with attachments this time
Date:	Wednesday, December 2, 2020 10:06:49 AM
Attachments:	clip_image001.png Revive Restore Chapter 1085.1 Comment.pdf PLOS_Horseshoe Crab copy.pdf

---

From: Ryan Phelan <ryan@reviverestore.org>
Sent: Thursday, November 26, 2020 6:23 PM
To: Leslie Furr <leslie.furr@USP.org>
Subject: Comments on 1085.1----with attachments this time

(Attached also in formatted PDF- as well as PLOS article)

RE: <1085.1> Use of Recombinant Reagents in the Bacterial Endotoxins Test—Photometric and Fluorometric Methods Using Recombinantly Derived Reagents in PF 46(5)

Dear Ms. Furr,

This letter is in response to the draft Guidance posted 01-Sep-2020 in PF 46(5) for a new General Chapter <1085.1>, *Use of Recombinant Reagents in the Bacterial Endotoxins Test—Photometric and Fluorometric Methods Using Recombinantly Derived Reagents.*

Revive & Restore is a non-profit organization bringing the tools of biotechnology to pressing conservation problems. One of these problems, of which you're fully aware, is the continued capture and industrial bleeding of the horseshoe crab (HSC), *Limulus polyphemus* to supply Limulus amebocyte lysate, or LAL for biomedical endotoxin testing. While this test is absolutely vital to global public health—especially during the Covid-19 pandemic and attendant development of injectable therapies and vaccines worldwide—the reliance on a threatened keystone species to supply LAL could be significantly reduced with greater adoption of recombinant factor C, or rFC.

Chapter <1085.1> is flawed by design, scope, and reach

- The proposed chapter <1085.1> increases barriers to rFC implementation, potentially delays approval of new drug products, and in effect will prevent the further adoption of rFC.
- The proposed comparability testing imposes an unnecessary burden of proof on pharmaceutical manufacturers and is duplicative with the FDA Guidance on Analytical Procedures and Methods Validation for Drugs and Biologics.
- The additional work USP is proposing to demonstrate comparability of rFC to LAL (a less reproducible assay that is subject to false positives) is an improbable task and one that appears to be based less on biological facts and more for a preference of maintaining a suboptimal status quo.
- This proposed chapter appears to be heavily influenced by LAL producers who may benefit from the USP raising deterrents to rFC use. The section on supplier, which is out of scope with USP's purview, also provides further advantages to LAL producers.

In lieu of Chapter 1085.1, we recommend instead a revision to Chapter <85> on Bacterial Endotoxins Test to include rFC as a named compendial method (or a new chapter numbered below <1000> without burdensome validation requirements.

- We advocate for a harmonized guidance for BET testing

We suggest revising compendial chapter <85> in a manner that would follow the EMQD in creating a stand-alone chapter to direct the use of rFC, with the ultimate goal of harmonizing both with Ph.Eur. 2.6.32 and <85>

- Regulatory authorities around the world have reviewed and approved the drug Emgality® in over 30 markets including in the U.S., Europe, Australia, Brazil, Canada, Israel, Kuwait, Lebanon, South Korea, Switzerland, Taiwan, United Arab Emirates. This approval, and discussions regarding future products have demonstrated that global regulatory scientists are generally favorable toward the use of rFC. This is no time for the US[hs1] [RP2] P to fall behind.

- Harmonization will help improve human health security worldwide.

Including rFC in chapter <85> will give pharmaceutical manufacturers a choice in BET testing, which means more options to a secure supply chain and better support for public health

- A revision to <85> would not *require* that the biomedical industry use of rFC, but would remove undue barriers to its adoption outlined in chapter <1085.1> and provide the biomedical industry with a second, sound option.

Reliance on the natural extract of a threatened species, during a time in which the capacity for drug development and manufacturing must increase significantly, jeopardizes public health. Coupled with climate unpredictability and the biodiversity crisis, there is a real risk that LAL supply will not meet demand.

A revised chapter <85> should acknowledge that the synthetic, rFC, is proven equivalent or superior to LAL

- It is critical to recognize that rFC represents an innovative technological advancement using the clotting cascade found in LAL. Based on fundamental modern recombinant biotechnology principles, rFC is equivalent to LAL. The recombinant Factor C protein is the clone of the horseshoe crab's natural Factor C, the bacterial endotoxin biosensor. The rFC assay employs the Factor C biosensor and uses the same Reference Standard Endotoxin calibrator for the same assay readout: the detection of endotoxin expressed in Endotoxin Units (EU).

- Current peer-reviewed literature exists that covers a broad specificity of rFC toward a wide range and diversity of endotoxins. In August 2020, the PDA published a review of the existing literature by a panel of industry, regulatory, and compendia bacterial endotoxins subject matter experts and concluded that rFC is comparable to LAL. In 2018, Revive & Restore published a review of the existing literature in the peer-reviewed journal *PLOS*. Our review concluded that rFC offers an equally effective, more consistent BET test than LAL.

- Nearly all the cited literature establishes comparability or demonstrates that rFC is equivalent

or superior to LAL.

Revive & Restore will continue to be an active and vocal proponent of rFC and the changes outlined above.

Thank you for your consideration in this matter.

Sincerely,

Ryan Phelan
Executive Director and Co-founder
Revive & Restore
ryan@reviverestore.org
415-710-9409 cell

revive&restore
genetic rescue for endangered and extinct species

November 25, 2020
Ms. Leslie Furr
Associate Scientific Liaison
The United States Pharmacopeial Convention, Inc.
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leslie.furr@usp.org

RE: <1085.1> Use of Recombinant Reagents in the Bacterial Endotoxins Test—Photometric and Fluorometric Methods Using Recombinantly Derived Reagents in PF 46(5)

Dear Ms. Furr,

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In lieu of Chapter 1085.1, we recommend instead a revision to Chapter <85> on Bacterial Endotoxins Test to include rFC as a named compendial method (or a new chapter numbered below <1000> without burdensome validation requirements.

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- We suggest revising compendial chapter <85> in a manner that would follow the EMQD in creating a stand-alone chapter to direct the use of rFC, with the ultimate goal of harmonizing both with Ph.Eur. 2.6.32 and <85>

- Regulatory authorities around the world have reviewed and approved the drug Emgality® in over 30 markets including in the U.S., Europe, Australia, Brazil, Canada, Israel, Kuwait, Lebanon, South Korea, Switzerland, Taiwan, United Arab Emirates. This approval, and discussions regarding future products have demonstrated that global regulatory scientists are generally favorable toward the use of rFC. This is no time for the USP to fall behind.

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- Reliance on the natural extract of a threatened species, during a time in which the capacity for drug development and manufacturing must increase significantly, jeopardizes public health. Coupled with climate unpredictability and the biodiversity crisis, there is a real risk that LAL supply will not meet demand.

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- Nearly all the cited literature establishes comparability or demonstrates that rFC is equivalent or superior to LAL.

revive&restore
genetic rescue for endangered and extinct species

Revive & Restore will continue to be an active and vocal proponent of rFC and the changes outlined above.

Thank you for your consideration in this matter.

Sincerely,

Ryan Phelan
Executive Director
Revive & Restore
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PLOS BIOLOGY

PERSPECTIVE

Saving the horseshoe crab: A synthetic alternative to horseshoe crab blood for endotoxin detection

Tom Maloney[1]*, **Ryan Phelan**[1], **Naira Simmons**[2]

1 Revive & Restore, Sausalito, California, United States of America, **2** Wilson Sonsini Goodrich & Rosati, San Francisco, California, United States of America

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Abstract

Horseshoe crabs have been integral to the safe production of vaccines and injectable medications for the past 40 years. The bleeding of live horseshoe crabs, a process that leaves thousands dead annually, is an ecologically unsustainable practice for all four species of horseshoe crab and the shorebirds that rely on their eggs as a primary food source during spring migration. Populations of both horseshoe crabs and shorebirds are in decline. This study confirms the efficacy of recombinant Factor C (rFC), a synthetic alternative that eliminates the need for animal products in endotoxin detection. Furthermore, our findings confirm that the biomedical industry can achieve a 90% reduction in the use of reagents derived from horseshoe crabs by using the synthetic alternative for the testing of water and other common materials used in the manufacturing process. This represents an extraordinary opportunity for the biomedical and pharmaceutical industries to significantly contribute to the conservation of horseshoe crabs and the birds that depend on them.

Citation: Maloney T, Phelan R, Simmons N (2018) Saving the horseshoe crab: A synthetic alternative to horseshoe crab blood for endotoxin detection. PLoS Biol 16(10): e2006607. https://doi.org/10.1371/journal.pbio.2006607

Published: October 12, 2018

Funding: The authors received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: BPOG, BioPhorum Operations Group; LAL, Limulus amebocyte lysate; LPS, lipopolysaccharide; rFC, recombinant Factor C.

Introduction

The 450 million-year-old horseshoe crab has been integral to the safe manufacturing of vaccines, injectable medications, and certain medical devices. Populations of all four extant species of horseshoe crab are in decline across the globe, in part because of their extensive use in biomedical testing [1]. From annual population surveys, it is clear that the biomedical industry's dependence on the horseshoe crab in North America is ecologically unsustainable. A synthetic alternative has been commercially available for more than a decade, and it has been unclear why the pharmaceutical industry continues to rely on the horseshoe crab. To answer this question, Revive & Restore, a nonprofit organization focused on protecting endangered species, researched the industry and interviewed industry experts [1].

In this paper, we synthesize 10 studies validating the efficacy of the synthetic alternative. Our review dispels lingering misconceptions and highlights the opportunity for the pharmaceutical industry to immediately embrace a new detection technology for common manufacturing materials, which will reduce the need to bleed horseshoe crabs by 90%. Phased

adoption will build the empirical data to confirm that synthetic endotoxin detection methods can be safely implemented, ending the industry's dependence on animal-based technologies. This transition is a critical step in turning the tide for the horseshoe crab and for the migratory birds that rely upon them.

Importance of the horseshoe crab to the pharmaceutical industry

In the United States, every drug approved by the US Food and Drug Administration (FDA) must be tested for bacterial contaminants. Endotoxins, common and potentially dangerous contaminants present in the outer membrane of the cell wall of gram-negative bacteria, can cause life-threatening fever or toxic shock if introduced intravenously, making their detection an essential safety test for the safe manufacture of all injectable medications [2].

For 40 years, from the 1940s to the 1970s, the pharmaceutical industry relied on rabbits to detect endotoxins, and hundreds of thousands of rabbits were euthanized annually. But by 1970, a new technique for endotoxin detection had been developed, using the blood of the horseshoe crab. It had been discovered that the horseshoe crab had a primitive but highly sensitive immune response to endotoxin contamination [3]. This primitive defense system is expressed in amebocyte cells, which circulate through the horseshoe crab hemolymph. The amebocytes are extremely sensitive to the lipopolysaccharide (LPS) found in endotoxins. When hemolymph comes into contact with gram-negative bacteria or LPS, the amebocytes begin to degranulate, and hemolymph coagulation is initiated by the granule components [4]. The reaction between the amebocyte and bacterial contaminants is the basis of the Limulus amebocyte lysate (LAL) test—the current standard for endotoxin testing around the world [5,6]. When implemented 40 years ago, the transition to the more efficacious LAL represented an incremental step away from laboratory animal-based endotoxin detection technologies, relying instead on blood from wild horseshoe crabs. Today, all injectable medications—as well as some environmental samples and medical devices—are screened for endotoxin contamination using the LAL test.

To create the LAL test, horseshoe crabs are captured and bled (Fig 1). The blood cells are then centrifuged and lysed in distilled water to release the cascade of enzymes responsible for recognizing endotoxins. To test a sample for endotoxins, a sample is mixed with the lysate at a specified ratio. Generally, the product of this reaction is detected as a gel clot, but it can also be successfully detected with chromogenic and turbidimetric techniques [7].

The horseshoe crab–derived test can be made from any one of the four extant species of horseshoe crab: *Tachypleus tridentatus* in Asia, *T. gigas* in Southeast Asia, *Carcinoscorpius rotundicauda* in Southeast Asia, and *Limulus polyphemus* in the Atlantic Ocean off North America [8].

Ecological impact of biomedical bleeding

Each year on the East Coast of the US, the biomedical industry captures approximately 500,000 horseshoe crabs and drains as much as a third of their blood. On average, 13% of the bled crabs are sold as bait for other fisheries, according to the Atlantic States Marine Fisheries Commission, while the rest are returned to the ocean [9]. Because horseshoe crabs are aquatic animals, the time out of water, combined with the bleeding process itself, is a significant source of injury and potential mortality. Conservation groups estimate the mortality rate of released crabs to be at least 15% to 30%. Regulators of regional fisheries use the 15% figure to guide management. Between the crabs sold for bait after bleeding and conservative estimates of mortality resulting from bleeding, at least 130,000 horseshoe crabs are killed annually by the biomedical industry.

Fig 1. Horseshoe crabs are bled at the Charles River Laboratory in Charleston, South Carolina, US. Photograph by Timothy Fadek.

https://doi.org/10.1371/journal.pbio.2006607.g001

Multiple studies have shown that during the weeks following bleeding, horseshoe crabs also experience detectable sublethal effects such as injury and disorientation [10,11], which lead to increased incidence of disease and possibility to lower spawning rates. The long-term effects of the bleeding procedure on breeding fitness are not yet understood.

Characterization of the LAL test derived from horseshoe crabs

The importance of endotoxin detection lead to an extensive characterization of the enzymatic components in the LAL endotoxin recognition cascade [12]. The LAL cascade is based on three kinds of serine protease zymogens—factor C, factor B, and proclotting enzyme z—plus coagulogen, a clottable protein. Endotoxins, notably LPS, activate the zymogen factor C to the active form, factor C [13,14,15]. Factor C then activates factor B to active factor B, which in turn converts the proclotting enzyme to the clotting enzyme. Each activation proceeds by limited proteolysis. The resulting clotting enzyme cleaves two bonds in coagulogen, which is a fibrinogen-like molecule in arthropods such as the horseshoe crab, to yield an insoluble coagulin gel [16]. The first molecule in the cascade, factor C, is the key molecule responsible for initiating the coagulation cascade system in the horseshoe crab hemolymph.

The development of a synthetic alternative

In 1997, scientists at the National University of Singapore, Ling Ding Jeak and Bo How, realized the potential that cloned, laboratory-synthesized recombinant Factor C (rFC) could have

for the development of an animal-free endotoxin detection technology. They were the first to clone the DNA of a factor C molecule and synthesized rFC, the synthetic alternative to the LAL test [17,18]. In contrast to LAL, the synthetic alternative utilizes a single protein cloned from a horseshoe crab as its active ingredient [19]. Subsequently, other groups cloned factor C molecules from different horseshoe crab species and studied them carefully to develop various rFC tests [8,20,21,22,23]. In the rFC test, the binding of endotoxin activates the synthetic rFC molecule, which then cleaves a fluorogenic substrate, resulting in the generation of a fluorogenic compound. The fluorescence is measured twice, first at time zero and then after the endotoxin has been introduced. The difference in fluorescence is proportional to an endotoxin concentration in the sample and is used to calculate a final endotoxin result.

Despite the optimism that the original development of rFC and similar recombinant technologies would largely displace the use of LAL, this did not occur. One commonly cited reason for the poor adoption was concern over the efficacy of rFC when compared to LAL.

Resolving questions about efficacy of the synthetic alternative

We reviewed multiple studies that evaluated the efficacy of rFC as an endotoxin detection method, summarized in Table 1. These studies tested a variety of different samples for endotoxin contamination. For the detection of gram-negative bacterial endotoxin, the rFC-based assay proved to be equivalent to the LAL test both in its ability to quantifiably measure endotoxin and in its ability to detect endotoxins across a range of concentrations. Furthermore, rFC is specific to endotoxin detection, whereas LAL is a nonspecific test: peptidoglycan from gram-positive bacteria, exotoxins from group A Streptococci, simple polysaccharides including yeast mannans and bacterial dextrans, and dithiols all activate LAL to give false positive results [24]. Notably, rFC does not contain glucan-sensitive factor G, meaning the rFC-based assay is not subject to false positives like the LAL test is.

Each of these 10 studies demonstrated that commercially available rFC tests detect endotoxins with results equivalent to or better than LAL, regardless of which company manufactured it. The breadth of these studies also showed strong efficacy across a range of uses and

Table 1. List of reviewed rFC efficacy studies.

Author(s)	Affiliations	Publication
Abate and colleagues [25]	Plymouth University University of Bristol University of the West of England	July 2017
Bolden and Smith [17]	Eli Lilly, Lilly Corporate Center, Indianapolis	July 2017
Schwarz and colleagues [26]	University of Salzburg	Dec 2014
Reich and colleagues [27]	Hyglos GmbH	June 2014
Chen and Mozier [28]	Pfizer, Inc.	March 2013
Grallert and colleagues [29]	Hyglos GmbH	Oct 2011
McKenzie and colleagues [30]	University of Massachusetts Harvard School of Public Health	May 2011
Thorne and colleagues [31]	University of Iowa Colorado State University	August 2010
Loverock and colleagues [18]	Lonza Walkersville, Inc.	Nov 2009
Bolden and colleagues [32]	Eli Lilly, Lilly Corporate Center, Indianapolis Genentech Bayer HealthCare LLC	July 2017

Abbreviation: rFC, recombinant Factor C.

https://doi.org/10.1371/journal.pbio.2006607.t001

demonstrated high sensitivity, strong reliability, and other positive considerations in the clinical use of rFC.

Sensitivity

The rFC assay demonstrated both a high rate and reliable sensitivity (in picogram quantities) of endotoxin detection for a variety of LPS structures [25]. The study conducted by Abate and colleagues found that, even at low amounts, the synthetic alternatives detected endotoxins with a wide range of structures. This is important because Lipid A from *Escherichia coli* LPS typically has a hexa-acyl structure, while LPS from different gram-negative bacteria may have different numbers and arrangements of acyl chains [25]. This is the first study to demonstrate that rFC assays can detect these various assay structures. This is significant in terms of reassuring laboratory personnel that rFC products exhibit strong sensitivity.

Range of applicability

Exposure to endotoxin poses a potential health risk in diverse clinical and nonclinical settings. Therefore, several of the studies listed above sought to confirm the suitability of rFC as an endotoxin assay in a variety of settings. For instance, Reich demonstrated that three commercially available synthetic reagents showed a 94.4% correlation to each other when testing water from a variety of sources, including lakes, springs, tap water, mineral water, and deionized water [27]. Also, a comparison of LAL and rFC for the assessment of airborne endotoxins found that the LAL and rFC assays are similar to air samples drawn from a variety of agricultural environments and over a wide range of concentration [30]. Furthermore, both methods yielded few nondetectable values. In a manufacturing setting, Chen and Mozier tested 13 therapeutic protein solutions at various stages of manufacturing and confirmed Schwarz's findings regarding the viability of rFC as a replacement for LAL.

Reliability

An important outcome of two of the studies above was that rFC demonstrates a higher rate of specificity for endotoxin [17,27]. As previously mentioned, LAL is not specific for endotoxin detection. It is well known that LAL testing cross-reacts with several β-glucans. Common sources of glucans include fungi (or yeast hydrolysate). Other sources, also ubiquitous in pharmaceutical manufacturing, are filters and other products made from cellulose materials, plant-derived raw materials, cotton-containing enclosures, sugars, and other naturally derived raw materials.

In addition to the issues caused by the presence of β-glucans, false positives can also be caused by proteases or phospholipids. There are no known false positives currently reported for rFC-based tests. Furthermore, buffers or solvents have been known to inhibit the sensitivity of LAL resulting in potential false negatives. Grallert and colleagues found that rFC overcame other sources of unreliable results that occur during LAL testing, including inhibitory constituents of the sample; fewer invalid results, which necessitate retesting; less interference in complex samples; and a broad dynamic range of 0.05 endotoxin units per milliliter (EU/ml) to 500 EU/ml [29].

Another reliability factor to consider is the lot-to-lot variability of the reagent used to detect endotoxin. One study that tested four different extraction and assay media concluded that on issues of lot-to-lot variability, the results of the rFC assay kits were superior to those previously reported for LAL assays [29]. Any variability encountered was easily overcome by using a standardized protocol for each test.

BioPhorum Operations Group study

In perhaps the strongest study, the BioPhorum Operations Group (BPOG), an industry consortium of biopharmaceutical manufacturers, formed a workgroup to develop a harmonized study design for assessment of endotoxin recovery with LAL, rFC, or control tests. Fourteen biopharmaceutical manufacturers, including Amgen, AstraZeneca MedImmune, Bayer, Biogen, Bristol-Myers Squibb, Eli Lilly, Johnson & Johnson Janssen, Lonza, Merck & Co. (USA), Merck Serono, Regeneron, Roche/Roche Genentech, and Sanofi/Sanofi Genzyme, performed experiments using similar protocols to determine if multiple laboratories would reach similar conclusions in detecting endotoxins with both LAL and rFC reagents. The study (hereinafter BPOG study) was jointly authored by laboratory heads at Eli Lilly, Genentech, and Bayer. They describe a robust, large-scale evaluation of both reagent types under different conditions, with tests conducted in 21 different biopharmaceutical laboratories [32]. In total, the study compared 37 different LAL/rFC reagent and supplier combinations. The study evaluated the three principal LAL test methods: the gel-clot, turbidimetric, and chromogenic methods. The study also evaluated the performance of rFC against the legacy LAL diagnostics.

The results of the BPOG study are striking. Although some variability was observed between tests, the BPOG study demonstrated that rFC can successfully detect naturally occurring endotoxins with a high limit of detection and in the presence of test "inhibitors," such as sodium citrate buffers.

Importantly, these data suggest that rFC is at least comparable, if not better than LAL in detecting endotoxins under various buffer conditions. The BPOG study further outlined a harmonized protocol that yielded consistent results across many different laboratories, regardless of whether the test was conducted with naturally occurring endotoxins or purified LPS. Importantly, the BPOG study demonstrated that effective and consistent results can be obtained with existing commercially available rFC reagents.

Removing barriers to the adoption of rFC

Although there is now abundant evidence that the efficacy of rFC is equivalent to or better than LAL in the detection of endotoxin, adoption of new technology is difficult, and change has come slow to the industry. The rFC assay has been commercially available since 2003, yet the pharmaceutical industry has been hesitant to utilize the synthetic alternative for a number of reasons. Endotoxin is a serious health concern; manufacturers and regulators have been justifiably cautious in the adoption of new detection technologies. Because endotoxin testing is highly regulated, pharmaceutical manufacturers have been inclined to follow known methods even when there is an opportunity to innovate.

Because vaccines and drugs are manufactured and distributed worldwide, different regulatory bodies (e.g., US FDA) rely on various compendia (e.g., US Pharmacopeia) and, where possible, a harmonization process to assure uniformity in endotoxin testing methods across all regulatory jurisdictions. In 2012, the FDA issued separate guidance acknowledging the use of rFC as an acceptable alternative to LAL, and the European health ministry followed. But because the use of rFC testing methods have not been incorporated into the harmonized Pharmacopeias, manufacturers must go through the extra step of validating the rFC assay, which is a more burdensome process than the streamlined method of verification used for methods described in the general Pharmacopoeia.

Leadership from the pharmaceutical industry that demonstrates a willingness to modernize laboratory processes and to covert to rFC is essential. Just by converting the testing of water and other common manufacturing materials, 90% of the demand for LAL in large-scale pharmaceutical manufacturing could be displaced, according to endotoxin experts with decades of

experience. There is a regulatory distinction between in-line processing and the final testing of the marketable drug product. Most manufacturers have the discretion to convert the testing of common in-line processing materials, such as pharmaceutical-grade water, without an onerous regulatory change process. Furthermore, increased utilization would advance the inclusion of rFC into the harmonized Pharmacopeias and would encourage other pharmaceutical companies to do the same. Until this year, rFC was under an exclusive patent, and pharmaceutical companies were reluctant to rely on a sole rFC supplier for such an important step in the manufacturing process. Regulators too were concerned about endorsing a method only available from a single manufacturer. Today, there are multiple suppliers, and more are expected to enter the market. Pricing is competitive with horseshoe crab-derived products and is likely to become even more advantageous with increased competition pricing resulting from new suppliers entering the field.

Horseshoe crabs are essential to a healthy ecosystem

In North America, compounding the threats of biomedical bleeding are fisheries pressures and the effects of climate change and rising sea levels, which are diminishing the availability of suitable spawning sites. The current overexploitation of horseshoe crabs is not dissimilar to other mismanaged species that have been driven to extinction. In 2016, the International Union for the Conservation of Nature [33] moved the mid-Atlantic populations of the American horseshoe from "near threatened" to "vulnerable" on its red list assessment. This reinforces the urgency for the biomedical industry to do its part to abate a major threat to the species by adopting synthetic alternatives and ending the use of LAL.

In the mid-Atlantic region of North America, the overharvest of the horseshoe crab is causing significant ecosystem-level impacts. Six species of shorebirds synchronize their northward migration along the Atlantic flyway to gorge on the eggs of spawning horseshoe crabs in Delaware Bay, a critical food stop on their journey to Arctic nesting grounds. Recent research has demonstrated that the abundance of horseshoe crab eggs is vital to both the survival and successful breeding of the birds that rely on them, particularly the red knot (*Calidris canutus rufa*), whose 9,500-mile migration from the tip of South America to the Arctic is among the longest of any bird in the world [34,35,36]. In 2014, a dwindling horseshoe crab population in North America prompted the classification of the red knot as threatened under the US Endangered Species Act [37]. The long-distance migratory birds that depend upon horseshoe crab spawning are some of the most rapidly declining shorebirds in North America.

Conclusion

The proven efficacy of the recombinant alternative for endotoxin detection provides an opportunity for the pharmaceutical industry to modernize procedures and contribute significantly to the conservation of horseshoe crabs. The move from rabbits to crabs occurred in the late 1970s; it is now time for the industry to modernize its methods and embrace a more humane and ecologically sustainable method of endotoxin testing. Immediate conversion to rFC for the testing of water and other common manufacturing materials presents no risk of diminution in reliability or sensitivity in endotoxin detection and is enabled under current regulatory guidance. Furthermore, based on interviews with industry experts, rFC presents advantages to LAL beyond the urgently needed benefits to conservation. Use of rFC largely eliminates the occurrence of false positive reactions to glucans and other commonly encountered substances. The reagent is more consistent since it is not subject to the lot-to-lot variability found in LAL. Importantly, industry experts have confirmed that conversion to rFC presents potential cost

savings, and these are expected to become more significant now that patent protections have expired and more rFC manufacturers are expected to enter the market.

Conversion to rFC would result in a 90% reduction in the demand for LAL, which means that mortality resulting from bleeding would decrease by an estimated 100,000 horseshoe crabs annually in North America alone. The relative threat abatement of widespread conversion to rFC for the three species of Asian horseshoe crab is hard to quantify, but any threat reduction will be beneficial for these species.

Horseshoe crabs face multiple threats, and the need for global conservation provides a remarkable opportunity for the biomedical industry to contribute significantly to their conservation. Given the equivalent efficacy, proven reliability, a clearly defined regulatory pathway, and the profound ecological benefits of ending the bleeding of horseshoe crabs, the authors recommend rapid proactive adoption of the recombinant-based alternatives as the standard method for endotoxin testing in pharmaceutical and biomedical laboratories worldwide.

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Boehringer Ingelheim

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November 30, 2020

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RE: General Chapter <1085.1> Use of Recombinant Reagents in the Bacterial Endotoxins Test—Photometric and Fluorometric Methods Using Recombinantly Derived Reagents

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Dear Ms. Furr:

We are writing in response to the draft Guidance posted 01-Sep-2020 for a new General Chapter <1085.1>, Use of Recombinant Reagents in Bacterial Endotoxins Test.

As you may know, our company has been working successfully since 2019 with recombinant factor C (rFC) as an equivalent alternative to the biologically sourced Limulus amebocyte lysate (LAL) assay. We believe that rFC is equivalent and comparable to LAL. Our company has extensively tested (process water, drug components,). We have found that rFC is equivalent to LAL in detecting bacterial endotoxins in injectable pharmaceuticals and is advantageous for us and for other manufacturers to use in ensuring the safety of our products.

Thus we were dismayed, first, when you abandoned progress toward revising the compendial chapter <85>, and second, instead of following the EMQD in creating a stand-alone chapter to direct the use of rFC (with the ultimate goal of harmonizing both with Ph.Eur. 2.6.32 and <85>), you instead created a guidance chapter that creates more, not fewer, barriers to adopting rFC than if you had left the status quo unchanged.

Unfortunately, the USP action adds unnecessary delay to incorporating rFC in a USP General Method. We hope that USP will engage all stakeholders equally and transparently to create solutions to bridge this gap in timing. We would much prefer that you move directly to a General Chapter similar to the European Pharmacopeia Ph.Eur. 2.6.32, either by amending <85> or creating a new sub-1000 chapter, e.g. <86>. But if you are determined to move forward with <1085.1>, we feel obliged to point out significant problems with the proposed chapter that must be resolved before it reaches its final form.

Most importantly, the proposed comparability guidance goes beyond FDA guidelines and published evidence for comparability and equivalence of the assays. Indeed, the additional work you are now requiring emerges not from weaknesses in rFC, but from the variability of LAL due to its biological origins and multiple manufacturers, and because LAL is subject to a range of artifacts, not least the enhancement of positive signals by the presence of glucans. Indeed, one could conclude from your document that it is LAL that needs to be proven against rFC's high sensitivity, reproducibility simplicity, and elimination of false positives. In short, you are asking for rFC to achieve

comparability with a more complex, less reproducible assay that is subject to elusive false positives. Why?

Thus, we request that you do the following:

- Remove from the proposed chapter <1085.1> occurrences of additional validation proof beyond what is already stated in 2012 FDA Guideline and <1225>, specifically the proposed comparability guidance.

- Remove from the proposed chapter <1085.1> the PTC on supplier quality as that is out of scope of public health standards

- Establish in short order a sub <1000> standalone chapter similar to Ph.Eur. 2.6.32, that can ultimately be merged into <85>, and which, importantly recognizes the scientific validity and comparability of rFC as does the EMQD.

We look forward to engaging with you further on this issue and will reach out to discuss how these matters may be moved forward in discussions with you.

Sincerely yours,

Paige E. Mahaney
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Global Head Biotherapeutics Discovery Research
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November 30, 2020

Mr. Mario Sindaco
Executive Secretariat
The United States Pharmacopeial Convention, Inc.
12601 Twinbrook Parkway
Rockville, MD 20852

REF: 11-20-049-T

Dear Mr. Sindaco,

The FDA has the following comments regarding the proposed **General Chapter <1085.1> Use of Recombinant Reagents in the Bacterial Endotoxins Test – Photometric and Fluorometric Methods Using Recombinantly Derived Reagents,** which appeared as an In-Process Revision in Pharmacopeial Forum Vol. 46, No. 5.

The term "recommend" indicates comments that we consider to be critical for the committee to address. The term "suggest" indicates less critical comments.

ENTIRE CHAPTER

1. The word "autochthonous" is unclear. When (and if) this concept appears in the chapter, the word "autochthonous" should be replaced with a simpler word that is easily understood. Although some specific examples are included below, this comment applies to the entire chapter.

2. We recommend replacing "USP Endotoxin RS" with "USP Reference Standard Endotoxin (RSE)," which is the correct terminology.

INTRODUCTION

1. We recommend revising the second paragraph for accuracy and clarity. It is more accurate to say that the reagents are cloned zymogen proteases (rather than saying that they use cloned zymogen proteases) and that the reagents are cloned from horseshoe crab genes (rather than saying that they are derived from horseshoe crab species). Therefore, we recommend revising as follows: "The recombinant reagents described in this chapter are not sourced..., but rather they are cloned zymogen proteases that comprise all or part of the natural lysate reaction cascade. These recombinant proteins are currently cloned from genes from any of the three horseshoe...."

BACKGROUND

1. First paragraph:
    a. For clarity, we recommend revising the first sentence to indicate that a series of proteases are activated in a cascading manner. We recommend revising as follows: “...the hemolymph clotting process...is a result of the cascading activation of a series of zymogen proteases.”
    b. Editorial correction: In the second sentence, “in vitro” should be italicized.

2. Beginning of the second paragraph: We suggest changing “recombinant bacterial endotoxin test reagents” to “recombinant reagents” to keep the terminology consistent with that use for the other reagents.

3. Second and third paragraphs: Use of the phrases "constituent zymogen elements" and “constituent of the clotting cascade” in the second and third paragraphs is confusing because the fourth paragraph uses the phrase "cloned zymogen proteases" instead, which seems more appropriate. For clarity, consistent terminology and descriptions should be used. Therefore, we recommend the following revisions:
    a. Second paragraph, first sentence: Revise as follows: “...cloned from or more genes of the natural cascade.”
    b. Third paragraph, first sentence: Revise as follows: “Current recombinant...the rFC protease of the of the clotting cascade.”

4. Fourth and fifth paragraphs: For clarity, we suggest using consistent terminology to describe the use of rFC and rCR reagents. In one paragraph, the method is "performed as described in <85>" and in the other it is "performed in a manner consistent with <85>.”

5. Last bulleted list: The third bullet point refers to the “glucan pathway” but the term “Factor G pathway” is used elsewhere in the document. To avoid confusion, we suggest using a consistent term throughout the document.

ALTERNATIVE METHODS

1. This chapter does not clearly explain the concept that the alternative methods should be first validated without specific products to demonstrate equivalency or non-inferiority of the methods compared to the compendial methods. We recommend revising this section to explain that validation of alternative methods should start without specific products (drug, biological device, etc.), and that once this has been demonstrated the method suitability study (inhibition/enhancement) using the specific product would be performed as described in USP <85> *Bacterial Endotoxins Test*.

VALIDATION OF ALTERNATIVE METHODS

1. Entire section:
    a. This section is confusing because it mixes product-free method validation with product-specific method suitability studies. We recommend reorganizing this section accordingly.
    b. The endotoxin test methods described in USP <85> are biological test methods, not microbial test methods. Therefore, reference to USP <1223> *Validation of Alternative Microbiological Methods* in this chapter could confuse stakeholders. Additionally, the FDA Guidance for Industry *Pyrogen and Endotoxins Testing: Questions and Answers* (2012) references USP <1225> *Validation of Compendial Procedures*, as well as USP General Notices 6.30, but not USP <1223>. We understand that USP <1223> was included in the chapter to cover the comparability requirement, but comparability of alternative methods to compendial methods is also covered in USP General Notices (section *6.30 Alternative and Harmonized Methods and Procedures*) and is referred to as "equivalence." Therefore, we recommend the following:
        i. Remove references to USP <1223> in this chapter. Instead, reference USP <1225> and the USP General Notices section 6.30 *Alternative and Harmonized Methods and Procedures*, as appropriate, to be consistent with the references in the FDA guidance.
        ii. It is not clear whether the user would need to perform all studies in USP <1225> or only those that are highlighted in this proposed chapter. For clarity, we recommend revising this section to indicate the specific studies from USP <1225> (and USP General Notices 6.30) that are needed.
        iii. Using the term "equivalence" throughout this chapter instead of "comparability" to be consistent with General Notices 6.30 and the FDA guidance.

2. Second paragraph, including the numbered list:
    a. Numbered list, item 1:
        i. The meaning of "documented formal study" is not clear. For example, are there informal documented studies or formal undocumented studies? For clarity, we suggest defining "documented formal study" or use the term "study" without modifiers.
        ii. We recommend adding accuracy and precision, range, and limit of quantitation to the analytical testing requirements of USP <1225>.
    b. Numbered list, item 2: If the names of sections in referenced USP chapters shouldn't be italicized, change the font for the referenced section of USP <85>.

c. Numbered list, item 3:

i. We recommend deleting the first sentence. Preparing endotoxins from autochthonous manufacturing sources is not scientifically justified. It is not clear why autochthonous manufacturing sources must be used and where would these be obtained. The same applicant could use different manufacturing facilities containing different microbial flora, which raises the question of whether their equivalence studies would need to be redone periodically and/or redone when the flora changes.

3. Third paragraph, including the bulleted list: We recommend either deleting the bulleted list from the chapter altogether or discussing this information under the reagent supplier section. The way in which these items are prominently listed in the "Validation of Alternative Methods" section suggests that there is a substantial concern about each of these. In the context of this chapter, these considerations could be framed as aspects that manufacturers have had to consider for recombinant reagent development. Our rationale for the recommendation to either delete or move this information is as follows:

a. Many of the bulleted items seem more like considerations for those who manufacture and market recombinant reagents.

b. As written, it seems as if the user should investigate these things as part of their validation. It is not clear how the user would use the information in the bullets to make a choice on which vendor's reagent to buy. The details seem a bit overwhelming for a user trying to choose a reagent.

c. Some of these items are out of control of the user and/or the information is not available to the user (e.g., proprietary information regarding the formulation). If this list is moved elsewhere in the chapter, we recommend revising the first sentence as follows: "and, if available, could be considered when choosing reagent suppliers and preparing comparability protocols…"

d. The references supporting these bullets do not necessarily promote the specific item for which is it listed as a reference. Some of the references for these bulleted items were not specifically focused on studying the specific bulleted item. The papers may note the specific item and indicate how the item may have affected the assay with the recombinant reagent, but these references were not negative or cautious about the use of the recombinant reagents. Additionally, we note that some of the "current literature" referenced in the list is from 1992 and 1993. If the list is moved elsewhere in the chapter, we recommend revising the references if more recent and relevant information is available.

4. Fourth paragraph:
    a. We recommend deleting the third sentence ("It is suggested that users of recombinant reagents…") because USP is not responsible for recommending feasibility studies.
    b. We recommend deleting the fourth sentence because users would need to contact the applicable regional authority regarding specific data (in addition to the suitability and validation studies described in USP <85> and USP <1225>) that would be needed for the method validation package.

VALIDATION OF ALTERNATIVE METHODS - Preparatory Testing and General Notes

1. This section refers to the endpoint chromogenic assay for the rFC assay and to the kinetic chromogenic assay for the rCR assay. The rationale for limiting each assay to a particular test method is not clear (i.e., whether the limitation is due to the current technology or to the assay design). For instance, it is not clear whether the rCR assay could be analyzed using the endpoint chromogenic assay. We recommend including clarification for limiting rFC analysis to the endpoint chromogenic test method and for limiting the rCR assay analysis to the kinetic chromogenic test method.

VALIDATION OF ALTERNATIVE METHODS - Comparability

1. First paragraph: The meaning of the second sentence is confusing, and it is not clear what "autochthonous endotoxins" are or whether firms have autochthonous endotoxin samples from manufacturing sources. Therefore, we recommend deleting this sentence.

2. Second paragraph, second sentence:
    a. The sentence states that "...comprehensive data demonstrating comparability of recombinant methods to LAL lysates in compendial articles containing assayable levels of endotoxins activity from autochthonous endotoxins are not available or have not been published in the public domain." This statement could become outdated quickly as new information is published in the public domain. Therefore, we recommend the following:
        i. Remove this statement or modify the next sentence in this paragraph (e.g., "unless data are available…").
        ii. Add reference to review articles which summarize the equivalency data that is available in the public domain (e.g., Bolden J, et al. Currently Available Recombinant Alternatives to Horseshoe Crab Blood Lysates: Are They Comparable for the Detection of Environmental Bacterial Endotoxins? A Review. *PDA J Pharm Sci Technol*. 2020;74(5) 602-611 or the individual articles that are currently available).

b. Additionally, the meaning of "autochthonous endotoxins" is unclear. If the second sentence of this paragraph is modified rather than deleted, we recommend removing the term "autochthonous endotoxins" from the sentence ("…comprehensive data demonstrating comparability of recombinant methods to LAL lysates…).

3. Third paragraph: We strongly recommend removing reference to "autochthonous endotoxin" and revising the paragraph to read as follows: "Samples should be "spiked" with the current USP Endotoxin RS (RSE) or commercially prepared control standard endotoxins (CSE) during the *Test for Interfering Factors*. Comparability cannot be demonstrated by using only test articles that do not contain levels of assayable endotoxins." Our rationale for this recommendation is as follows:

    a. FDA relies on the use of RSE and CSE for these studies instead of unknown sources of endotoxins (e.g., "naturally occurring endotoxins" or "autochthonous endotoxins"). The suggestion to use unknown sources of endotoxins for comparability studies appears to revert what the FDA has recommended for decades. The inclusion of this paragraph will cause confusion and lead to sponsors having to repeat studies using the appropriate standards at the FDA's request.

    b. The suggestion to use "autochthonous endotoxins" for comparability studies conflicts with the subsequent suggestion in this section (numbered list, item 2) to use RSE instead of CSE, as "autochthonous endotoxins" would be even more variable than CSE, and it would not be reasonable to expect firms to have a panel of endotoxin contaminated samples from a manufacturing environment.

4. Fourth paragraph: We strongly recommend deleting the entire paragraph ("If manufactured drug product…eventually contaminate the product.") for the same reasons described above for the third paragraph of this section. Additionally:

    a. "Diluent containing a phylogenetically diverse population of endotoxins" is not clearly defined. It is not clear whether the statement is suggesting that the user prepare a cocktail of organisms, or how many organisms would fit the definition "phylogenetically diverse."

    b. The validation should be performed in context of the user's product and experience, and it seems unreasonable to ask the user to find and develop a diverse population of samples.

    c. Although the second sentence is prefaced with "historically," references 21 and 22 are from 1925 and 1945. Pharmaceutical company and municipal water systems have advanced substantially since 1925 and 1945. References this old should not be in this discussion on how to perform validation of the alternative method, as they don't represent cGMP. Upstream water is not relevant to contaminants found in a current manufacturing process. Additionally, Gram-

negative organisms can enter a manufacturing facility from other sources other than water systems.

5. Fifth paragraph:
    a. Numbered list, item 1: We recommend deleting this item because use of a glucan blocker may not eliminate all glucan present in samples.
    b. Numbered list, item 2:
        i. The fourth sentence suggests that comparability studies employ the USP Endotoxin RS for calibration curves and positive product control, but it does not clearly explain why RSE should be used instead of CSE. We recommend revising the sentence to more clearly explain that it would be best to use RSE because it would eliminate variability that could be introduced by using CSE.
        ii. Additionally, we recommend moving the fourth and fifth sentences to the beginning of item 2, followed by the rationale for using RSE instead of CSE for comparability studies.
    c. Numbered list, item 3, relative recovery calculation, and the sentence following the recovery calculation:
        i. We recommend removing the term “autochthonous” from the last sentence in item 3 (and editing the remainder of the item accordingly) for the reasons described above for the third and fourth paragraphs of this section. Additionally, the term “autochthonous” is confusing.
        ii. The relative recovery calculation is not explained accurately. We recommend explaining the following:
            1. The endotoxin value using recombinant reagents should be within 50-200% of endotoxin value using LAL; i.e., 5.3 EU/mL of recombinant endotoxin should be within 50-200% (2.35-9.4 EU/mL) of 4.7 EU/mL (LAL endotoxin).
            2. However, a range of 50-200% is very high and we strongly recommend that it should be more stringent i.e., 70-130%.

TEST PROCEDURE

1. Refer to the comment above under “Preparatory Testing and General Notes” regarding reference to the endpoint chromogenic assay for the rFC assay and to the kinetic chromogenic assay for the rCR assay.

INTERPRETATION

1. Refer to the comment above under “Preparatory Testing and General Notes” regarding reference to the endpoint chromogenic assay for the rFC assay and to the kinetic chromogenic assay for the rCR assay.

POINTS TO CONSIDER: SUPPLIER QUALITY

1. The supplier is responsible for characterizing and qualifying the reagent, while the user is responsible for using a reagent that meets quality standards (e.g., specific activity) and demonstrating that the reagent is fit for its intended use. Because this section focuses mostly on supplier responsibilities, we recommend changing the section header from “Points to Consider: Supplier Quality” to “Points to Consider for Reagent Suppliers.”

2. Second paragraph: We suggest changing “critical ingredients” to “raw materials,” which is the generally accepted term.

3. We recommend deleting Table 1 and presenting the user versus supplier responsibilities in a streamlined manner. Specifically:
    a. Replace the information in the first two rows of the table with a general statement about supplier versus user responsibilities (such as “the supplier is responsible for characterizing and qualifying the reagent, while the user is responsible for using a reagent that meets quality standards (e.g., specific activity and demonstrating that the reagent is fit for its intended use”) which could be added to one of the paragraphs above the table.
    b. Present the supplier responsibilities shown in the third row of Table 1 as points to consider for reagent manufacturers. This information could be presented as a bulleted list in the text. Additionally, we recommend the following changes to this information:
        i. Sentence above the bulleted list: The point of this section of the table is to ensure that changes in the reagents do not impact sensitivity. We recommend revising this sentence to read “Documentation and assurance that changes in the following elements do not affect the sensitivity or specificity of the reagent and that those changes are properly documented and controlled:” because this section doesn’t read correctly without the word “changes” included.
        ii. First bullet point: We recommend changing “optimal choice of species of the horseshoe crab as the source of the recombinant proteins” to “specific activity of the recombinant proteins” because it is the of the recombinant proteins that is important. The DNA sequences for the specific factor C or

cascade genes or proteins from the different crab species do not necessarily need to be 100% homologous to code for proteins with the same activity.

iii. Second bullet point: We recommend changing "optimal number of cascade zymogens present in the formulation" to "number of cascade zymogens present in the formulation." The change that we want to address is the change in the number of proteins, as the optimal number of zymogens would not change.

iv. Fourth bullet point: We recommend changing "identification and verification of any post-translational modifications" to "post-translational modifications of the recombinant proteins" because the change would be to the post-translational modifications (not to the identification and verification of modifications).

v. Longevity of rFC or rCR proteins (i.e., shelf-life) should be tested and compared with newly synthesized protein and the native protein to identify if recombinant protein is stable or more susceptible to degradation. We recommend including this as a supplier responsibility.

vi. Suppliers should assess the impact of process changes on reagent quality (stability, post-translational modifications, etc.). We recommend including this as a supplier responsibility.

We hope these comments will be helpful to USP and the General Chapters - Microbiology Expert Committee. Please feel free to contact Dr. Colleen Thomas on my staff at Colleen.Thomas@fda.hhs.gov if there are any questions. Please use the reference number provided above on any ensuing correspondence.

Sincerely yours,

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