Functional Challenges for Alternative Bacterial Endotoxins Tests Part 4: Beyond Recombinant Reagents

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Introduction

The two compendial tests, <151>, "Pyrogen Test" and <85>, "Bacterial Endotoxins Test" (henceforth "RPT" and "BET" respectively) share a common reason for their genesis: the quality defect once common in parenteral therapies known as "injection fever." Data collected over the last century confirm that contaminating Gram-negative bacterial endotoxins were the cause of injection fever and remain the most probable source of the vast majority of patient febrile reactions resulting from contaminated drugs, biologicals and medical devices.

Unfortunately, the terms "endotoxin" and "pyrogen" have come to be used interchangeably. This is understandable in the sense that "pyrogen" is defined as "A substance that elicits a febrile response" (Reference Table 1), a subset of which has been identified as bacterial endotoxins. These endotoxins are derived from the outer leaflet of the outer cell membrane of most Gram-negative bacteria. The introduction of alternate test methods to the compendial tests, particularly cell based methods, that purport to detect pyrogens (including endotoxins) require provision of a scientific, contemporary and consistent set of terms and associated standards to assure properly validated assays and continued patient safety.

Background

The early twentieth century saw a focus on understanding the causes and implications of "injection fever", the collective name for febrile responses often seen in patients receiving IV injections or infusions. This focus led to the observation that these "pyrogenic" substances were not filterable, were comparatively heat stable, and resulted from the proliferation of Gram-negative bacteria, primarily from the genus *Pseudomonas* that were present in the distilled water used to prepare parenteral solutions (Hort and Penfold 1911; Hort and Penfold, 1912; Seibert, 1923; Seibert 1925; Bourn and Seibert, 1925). This finding led to a test to screen parenteral products for pyrogenicity in order to reduce or eliminate the incidence of injection fever. It was the United States Pharmacopeia that undertook a study using a rabbit model to understand febrile responses induced by a range of dosing regimens of a cell-free preparation of *Pseudomonas aeruginosa*. Those data, termed "pyrogenic dose" were ultimately reported in 1943 (McClosky, et al) and the USP Pyrogen Test, performed in rabbits, was granted compendial status in 1942, with the intended purpose of screening drug products for "pyrogens", most notably endotoxins from Gram-negative bacteria.

In 1964, two researchers from Johns Hopkins University who were exploring marine animal models to study human disease published a seminal study describing the role of Gram-

negative bacteria in the extracellular coagulation of the blood of the North American Horseshoe Crab, *Limulus polyphemus* (Levin and Bang, 1964). Recognizing the value of using this clotting phenomenon for the detection of contaminating Gram-negative endotoxins often found in pharmaceutical products, James Cooper and co-workers at Johns Hopkins University published data on the quantitative comparison of the responses of the existing RPT and the new Limulus Amoebocyte Lysate (LAL) test in the detection of endotoxins in short-lived radiopharmaceuticals (Cooper, et al, 1971). Their study demonstrated that testing products using the LAL reagent, considered to be an alternative test to the RPT at the time, could detect endotoxins in pyrogenic preparations in 15 minutes, a significant advancement over the RPT to prevent the administration of contaminated radiopharmaceuticals to patients. Cooper and co-workers' data were the impetus for a fifteen year-long collaborative effort by industry, regulators, and clinicians to study the LAL test and compare its capabilities to the RPT to ascertain if the two assays were equivalent for the detection of bacterial endotoxins. This effort addressed a number of functional challenges: analytical capability, sample-specific method suitability, and comparability of test results for the two methods to detect contaminating endotoxins from autochthonous Gram-negative bacteria in relevant pharmaceutical matrices. The aggregate data obtained from many independent assessments and in many different parenteral formulations culminated in the publication of <85> "Bacterial Endotoxins Test" (BET) as a chapter in USP in 1982 that describes LAL-based methods, and the issuance of a Guideline by the US Food and Drug Administration for the use of LAL as a replacement for the RPT for the screening of drugs, biological products, and medical devices for the presence of pyrogens (1987, now retired).

The twenty-first century has re-focused attention on endotoxin and pyrogen testing to encourage the use of non-animal derived test methods or methods that require far less Limulus blood than has been historically used. Currently, there are several categories of commercially available methods that do not require live animal involvement. The majority of these methods utilize the USP Reference Standard Endotoxin as a calibration analyte and report results in Endotoxin Units (EU, a measure of endotoxins activity) or Endotoxin Unit Equivalents:

- ELISA assays including the Monocyte Activation Test (MAT)
- Toll Like Receptor Assays
- Recombinant cascade methods/ Recombinant Factor C

Table 1 provides a set of recommended terms and definitions that are salient to alternative test methods and the validation of alternate test methods.

Pyrogens and Endotoxins

Endotoxins, when injected into the human bloodstream, stimulate the release of cytokines via the innate mammalian immune system. Cytokine release may, depending on the potency and dose of endotoxin, result in a febrile reaction. While a complete list of pyrogenic materials conceptually includes many substances from

Table 1. A contemporary set of terms and definitions for endotoxin testing

Term	Definition
Alternative Test Method	An orthogonal test method to an official compendial method
Bacterial Endotoxins	See endotoxins
Bacterial Endotoxins Test	An official compendial method to detect Bacterial Endotoxins activity based on the clotting cascade of amoebocytes derived from blood of the horseshoe crab (Limulus Amoebocyte Lysate - LAL)
Calibration Analyte	The reference standard solution used to prepare standard curves and positive controls. Most pharmacopoeial endotoxin reference standards should be more correctly described as purified LPS since its chemical nature after purification is a lipid component called Lipid A, covalently bound to a polysaccharide composed of two parts, the core and a variable O-specific side chain, responsible for the specific immune reaction evoked in the host. (Franco et al, 2018)
Endotoxin	A high molecular weight complex that contains lipopolysaccharide (LPS), protein, and phospholipid originating from the outer membrane of Gram-negative bacteria. (Franco et al, 2018)
Equivalence	A two-sided statistical test designed to show that a test condition is neither better nor worse than a control condition
Febrile Response	A cytokine-mediated rise in body temperature
Pyrogen	A substance that elicits a febrile response
Pyrogenic/Pyrogenicity	Ability to elicit a febrile response
Pyrogenic dose	The amount of a substance relative to body weight that can elicit a febrile response after administration
Rabbit Pyrogen Test	A compendial method having the intended purpose of screening drug products for pyrogens, most notably endotoxins from gram-negative bacteria.

chemicals to viruses to bacteria, in the parenteral drug, biologic, or medical device manufacturing environments, endotoxin contaminants derived from water-borne Gram-negative bacteria adapted to the manufacturing materials and environment predominate and are also the most potent and well-studied pyrogens currently known (Pearson, 1985; Sandle, 2015; Akers, 2016; Dubczak, 2020).

While microbial substances other than endotoxins may be pyrogenic, they are generally substantially less potent than endotoxins. For example, the minimum pyrogenic dose of peptidoglycan in rabbits is 7.3 µg/kg (Martis, et al, 2005), which is estimated to be approximately 108 cells (Sandle, 2015.) In contrast, the mean pyrogenic dose of E. coli lipopolysaccharide (LPS, the active component of endotoxin) in rabbits is about 1.6 ng/kg (Dabbah, et al, 1980), suggesting that the LPS is almost four orders of magnitude more potent than peptidoglycan. Given the differential in potency compared to bacterial endotoxin, the peptidoglycan level measured in micrograms would be present only if pre-sterilization bioburden were exceptionally high, a circumstance that should be detected by an organization's GMP bioburden control program.

Whether an alternative assay is commercially available or developed in-house, or whether it purports to be an endotoxin assay or a pyrogen assay, it should be able to detect bacterial endotoxins, which to date remain the only well-studied source of pyrogens in injectable products. If a company, through risk analysis, identifies additional sources of potential pyrogenic responses such as peptidoglycan or flagellin, the pyrogen alternative assay must be able to demonstrate a quantitative recovery of these substances, as well as endotoxins, via product-specific suitability testing.

Three Part Approach to Validation of Alternative Cell-Based Methods

Previous publications in this series described a three-part approach to the validation of the use of recombinant reagents as alternative methods used in the performance of the BET: analytical capability, product-specific suitability, and comparability of the candidate method to the compendial method with respect to the quantitative recovery of endotoxins activity from microorganisms autochthonous to the manufacturing environment (Akers, et al., 2020a, 2020b, 2020c). The same challenges apply to all alternative methods including the currently available cell-based assays and any future biochemical analytical methods designed to detect and quantitate pyrogens, particularly bacterial endotoxins.

1. Any alternative assay must meet appropriate analytical requirements as described in USP <1225>

Although <1225> "Validation of Compendial Procedures" is not written specifically for the detection of biological contaminants, it is important that any assay used as an alternative to <85> or <151> demonstrates appropriate accuracy, precision, reproducibility, reliability and specificity. Consistent with <1223>, "Validation of Alternative Microbial methods", specificity is a method's ability to detect a range of pyrogens *specific to the technology's claim*. "Range of pyrogens" may be defined as a range of endotoxins from Gram-negative bacteria autochthonous to the manufacturing materials and environment. Alternatively, it may include other identified pyrogens such as peptidoglycan, flagellin or a specific chemical that may represent a risk to patient or product in addition to endotoxins. (Martis, et al, 2005; Hasiwa, et al, 2013)

The endotoxin unit (EU) is a measure of endotoxins activity, so defined after considerable testing based on the RPT (Mascoli and Weary, 1979; Dabbah, et al, 1980; Tsuji, et al, 1980;). The EU is the unit of measure of the Threshold Pyrogenic Dose (TPD), which is the numerator in the formula used for the calculation product-specific endotoxin limits. However, some alternative methods may not use currently defined endotoxins activity as the basic measure for pyrogenic content. If the alternative method reports test results in a unit of measure other than EU (or "EU Equivalent" then the correlation between safety and the proposed unit of measure, including how the candidate method relates to existing calculated endotoxin limits, must be determined. Comparability with respect to different analytical signals is discussed in <1223> "Validation of Alternative Microbiological Methods."

2. The user must be able to demonstrate method suitability with materials under test

It is important for each QC laboratory to demonstrate that the candidate method, when used to assay prepared test articles, does not create a bias in test results, meaning that the alternative method should not result in inhibition (underestimation) or enhancement (overestimation) of target pyrogenic activity. Unlike comparability testing, suitability is demonstrated using an analyte, generally an accepted pyrogen reference standard of known source and potency such as the USP Reference Endotoxin Standard to compare the level of activity added to the prepared material with the level of recovery in the test result.

3. The candidate method must demonstrate equivalency of test results with the compendial method including the detection of endotoxins from autochthonous Gram-negative bacteria found in the manufacturing environment

The intent of any current pyrogen assay (RPT or BET) is to detect, and in the case of BET, quantitate, unknown levels of activity of pyrogens from unknown sources. While these pyrogens are overwhelmingly endotoxins from a range of Gram-negative bacteria that may be found in the manufacturing environment, additional pyrogenic substances could be identified by the developer or user of the alternative method. *Because of their importance in the manufacture of parenteral products, the detection and quantitation of endotoxins from autochthonous Gram-negative bacteria must be a requirement for any alternative method.*

In 2008, the United States Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), including participation by Drs. Hussong and Mello, co-authors of this article, published a report on the *Validation Status of Five In Vitro Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and*

Other Products. The seven functional qualification recommendations made by ICCVAM for *in vitro* pyrogen methods (e.g. Monocyte Activation Test) are:

1. Both endotoxin-spiked and non-endotoxin spiked samples should be included. Non-endotoxin pyrogen standards should be characterized prior to their use in any study, if possible.
2. All aspects of the studies should comply with Good Laboratory Practices.
3. Future studies should include products that have intrinsic pro-inflammatory properties in order to determine if these tests can be used for such substances.
4. Optimally, a study that includes three-way parallel testing, with the *in vitro* assays being compared to the RPT and the BET, should be conducted to comprehensively evaluate the relevance and comparative performance of these test methods. These studies may be conducted with historical RPT data provided that the same substances (i.e., same lot) are tested in each method. Based on ethical and scientific rationale, any *in vivo* testing should be limited to those studies that will fill existing data gaps.
5. Test substances that better represent all categories of sample types (e.g., pharmaceuticals, biologicals, and medical devices) intended for testing by the methods should be included.
6. The hazards associated with human blood products should be carefully considered, and all technical staff should be adequately trained to observe all necessary safety precautions.
7. Formal sample size calculations should be made to determine the required number of replicates needed to reject the null hypothesis at a given level of significance and power. For reliability assessments, formal hypothesis testing is essential with the alternative hypothesis being no difference between groups.

These recommendations represent a rational and objective path forward for the validation of alternative cell and bioanalytical methods for compendial pyrogen and endotoxin testing. A number of caveats to the ICCVAM recommendations could be added:

- The use of endotoxins from autochthonous sources is essential to the demonstration of test result comparability
- For point #4, above, studies need not be conducted on consecutive batches of product, and may include the use of historical data (for example RPT data if it had been performed at the time of release) or stability samples of the test article to complete the required comparability tasks.
- For standard hypothesis tests (point #7, above), we must emphasize that failure to reject the null hypothesis does not imply equivalence.

Additional Challenges Facing Alternative Pyrogen or Endotoxins Testing

Regulatory requirements may raise additional challenges with respect to how comparability or equivalence to the Pyrogen Test might be demonstrated. In 2012, FDA published an updated question and answer document entitled "Guidance for Industry: Pyrogen and Endotoxins Testing: Questions and Answers" (FDA, 2012). In response to Question 9, which asks "When is the USP Chapter <151> Pyrogenicity Test appropriate?", the Guidance provides the following:

- *"For certain biological products, 21 CFR 610.13(b) requires a rabbit pyrogen test. The requirement in 21 CFR 610.13(b) may be waived if a method equivalent to the rabbit pyrogen test is demonstrated in accordance with 21 CFR 610.9.*
- *For human and animal drugs, some USP monographs still require a rabbit pyrogen test. Even with such monographs, a firm may substitute an endotoxins test or alternative cell-based test if the firm can demonstrate equivalent pyrogen detection. The appropriate FDA review division will consider alternative methods, such as monocyte activation, on a case-by-case basis.*
- *For devices and drug materials, firms should assess the risk of the presence of non-endotoxin pyrogens. If the risk assessment indicates that non-endotoxin pyrogens may be present, it may be more appropriate to use the rabbit pyrogen test.*
- *Bacterial endotoxins assays are subject to a variety of interferences related to the physical and chemical properties of the test article. Where such interferences cannot be mitigated through sample dilution (up to the MVD) or other validated means of sample preparation, firms should use the rabbit pyrogen test."*

Conclusion

There are many stakeholders who are interested in the evolution of new test methods for pyrogens in parenteral products and medical devices. There is also the safety of billions of patients, both human and veterinary, to consider. It is unproductive to be mired in 1970s thinking or demand tests that will increase costs with no established benefit to product quality or patient health. New alternative methods must be at least as good or better at assessing and ultimately assuring patient safety than the current compendial methods. Overcoming the functional challenges to implementing alternative pyrogen tests will require the continued commitment to scientific integrity, and the assurance of a primary focus on patient safety from industry, reagent/method suppliers, regulators, and the compendia.

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PYROSMART NEXTGEN™

PyroSmart NextGen™ is a sustainable recombinant Cascade Reagent (rCR) that delivers the same reliable results as your conventional LAL reagent and offers these additional advantages:

- **No Animal Content – Horseshoe Crab Blood Free**
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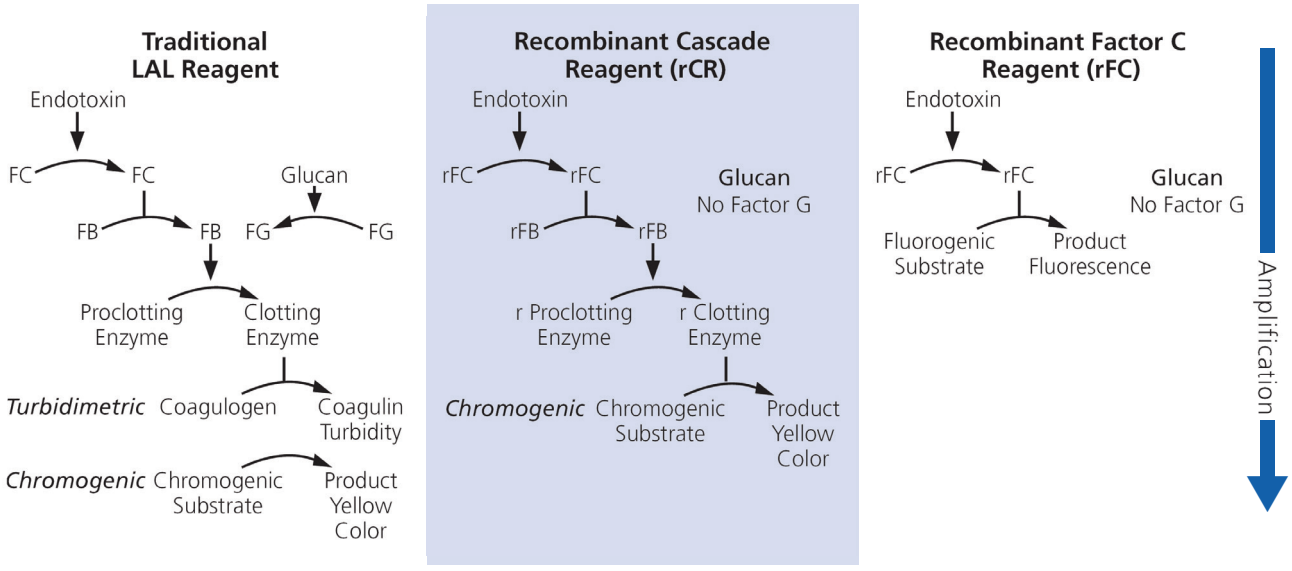
- No Cross Reactivity With 1,3-β-D-glucans
- Same Instrument
- Same Preparation Steps
- Meets Your Sustainability Objectives

Keep Your Method. Make An Impact.

ACC's PyroSmart NextGen™ uses the same cascade as traditional LAL reagents by manufacturing the Factors responsible for the cascade using recombinant processes. As a result, our new recombinant reagent's mechanism of action will deliver results consistent with traditional LAL reagents. It offers the added advantage of eliminating 1,3-β-D-glucans cross reactivity from the LAL cascade, since there is no Factor G in the final reagent.

ACC developed Pyrosmart NextGen™ to provide a sustainable alternative to traditional naturally sourced LAL reagents, while maintaining your lab procedures, methods, instrumentation and most importantly your results.

The Importance of Mechanism of Action Recombinant Cascade Reagent (rCR)



Traditional LAL reagent

In the presence of endotoxin, Factor C becomes an activated moiety which in turn activates Factor B and Proclotting Enzyme; ultimately resulting in the proteolytic cleavage of a substrate (either coagulogen in gel clot and turbidimetric assays or a colorless chromogenic substrate in chromogenic assays). The cascade mechanism thus amplifies the response of Factor C and leads to an exceptional sensitivity for this biological assay, with kinetic output being preferable. In the presence of 1,3-β-D-glucans, Factor G becomes an activated moiety which also activates Proclotting Enzyme and thus resulting in the same signal as that triggered by endotoxins through Factor C. This has been often observed as glucan-derived enhancement or false positive results.

Recombinant Cascade Reagent (rCR)

As with naturally sourced LAL reagents, in the presence of endotoxin, recombinant Factor C becomes an activated moiety which in turn activates recombinant Factor B and recombinant Proclotting Enzyme; ultimately resulting in the proteolytic cleavage of a colorless chromogenic substrate formulated with PyroSmart NextGen™. By relying on the same cascade mechanism, the response of recombinant Factor C is amplified the same way as by LAL reagents and thus the same sensitivity is achieved using this kinetic assay. Due to absence of Factor G, PyroSmart NextGen™ will not react with any 1,3-β-D-glucans and therefore will prevent glucan-derived enhancement and false positive results.

Recombinant Factor C (rFC) – Competition

Launched almost two decades ago, rFC reagents rely **only** on a recombinant form of Factor C. Due to the absence of the cascade as the amplification mechanism, rFC reagents are paired with a fluorescence method instead. However, this constitutes a different measured entity, different instrumentation, and different preparation steps with a limited output (endpoint assay only). Therefore the uptake and implementation of this method has been rather limited.

RECOMBINANT LAL REAGENT

The Benefits Are Clear

LAL Reagent Comparison Table	Conventional LAL Reagent	**ACC's PyroSmart NextGen™ (rCR) Reagent**	Competitor (rFC) Reagent
Year Technology Introduced	1977	**2021**	2003
Kinetic Assay	Kinetic	✓ Kinetic	✗ **No.** Endpoint only
Assay Setup	Single step reconstitution	✓ Single step reconstitution	✗ **No.** rFC requires three reagents in a 1:4:5 ratio and a 10 min. pre-incubation step
Same Standard Plate Reader	Incubating plate or tube reader at 405 nm	✓ **Yes.** Incubating plate or tube reader at 405 nm	✗ **No.** Fluorescent reader required
Derived From *Limulus* Amebocyte Lysate (LAL)	LAL	✓ **Yes.** rCR is recombinant LAL	✗ **No.** Based on *Carcinoscorpius* or *Tachypleus* Amebocyte Lysate (CAL/TAL)
Multi-step Cascade Pathway	Yes	✓ **Yes**	✗ **No**
Endotoxin Specific	No	✓ Endotoxin Specific	✓ Endotoxin Specific
Sustainable Reagent (animal free)	No	✓ Horseshoe Crab Blood Free	✓ Horseshoe Crab Blood Free

Converting to PyroSmart NextGen™ is Easy

Switching to this sustainable alternative is easy because PyroSmart NextGen™ follows the same cascade pathway as traditional reagents.

But don't take our word for it – evaluate PyroSmart NextGen™ yourself on your existing absorbance readers. Follow our user-friendly Evaluation Protocol to determine if PyroSmart NextGen™ works in your laboratory and on your samples. Our experts will assist you every step of the way.

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- **Same Preparation Steps**
- **Same Method**

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