American Pharmaceutical Review™

Endotoxin Detection Part IX:

Current Issues And Future Considerations for Pyrogen-Free Products

A Supplement to American Pharmaceutical Review

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Endotoxin Detection Part IX

Current Issues And Future Considerations for Pyrogen-Free Products

A Note from the Editor

Welcome to our ninth supplement on Endotoxin Detection.

Since the publication of our last supplement approximately one year ago, the pharmaceutical industry has done what was previously through impossible: brought to market not one – but several highly effective and safe vaccines for COVID-19.

This herculean task could not have been accomplished without a combination of government, regulatory, and industry professionals working together to serve a common goal.

In addition, the contribution from industry technology and service providers cannot go without mention. The breadth of tools, technologies and expertise these companies provide to the industry is truly staggering.

As we are all aware, the best therapeutic products can be rendered worthless if they are found to be contaminated.

As the number of biopharmaceutical products grow – the need to test for bacterial endotoxins also grows. And, as the need for these new drugs becomes more critical the need to ensure these products are free from endotoxins has never been more important. There simply is no room for error when producing new drugs – consumer expectations and the need to ensure consumer confidence are at an all-time high.

The goal of this supplement is to provide as much information as possible regarding current thinking and methodologies for endotoxin testing and removal. As indicated in the table of contents below there is much to be said on this topic – and we have enlisted the expertise of many of the industry's subject matter experts.

As you look through these articles we hope you gain valuable insight and knowledge regarding this industry critical topic.

If you have any questions or comments, please contact us.

Thanks again for reading,

Mike Auerbach
Editor In Chef

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CompareNetworks

395 Oyster Point Blvd. Suite 300
South San Francisco, CA 94080
info@aprpub.com
www.americanpharmaceuticalreview.com

ASSOCIATES OF CAPE COD INCORPORATED

Specialists in Endotoxin and Glucan Detection

Associates of Cape Cod, Inc.

124 Bernard E. Saint Jean Drive
E. Falmouth, MA 02536
Tel: 888-395-2221
Email: custservice@acciusa.com

www.acciusa.com

Specializing in chromogenic and turbidimetric reagent technologies, Associates of Cape Cod, Inc. (ACC) has been a global leader in endotoxin and (1→3)-ß-D-glucans detection products and services for over 45 years. ACC pioneered LAL testing methodology and was the first FDA licensed company to manufacture LAL reagents, and throughout the years has grown to be an internationally recognized leader in endotoxin detection.

Earlier this year, we were very excited to introduce another first when we launched the first and only commercially available sustainable BET reagent, PyroSmart NextGen™ - Recombinant Cascade Reagent (rCR). PyroSmart NextGen™ is completely horseshoe crab blood free and unlike first generation recombinant BET reagents (rFC), PyroSmart NextGen™ is the only one that uses the same LAL cascade as traditional LAL reagents, while eliminating the potential for 1,3-ß-D-glucans. Simply put… same Instrument, same preparation steps, same method. **Keep your Method… Make an Impact!**

Our worldwide headquarters are in East Falmouth, Massachusetts. With a dedication to quality, ACC is certified to I.S. EN ISO 13485:2016 and ISO 13485:2016. We are FDA Inspected and operate DEA Licensed and CLIA-certified laboratories. Our endotoxin detection reagents, instruments and software are used within the Pharmaceutical, Medical-Device, Biotechnology, Compounding Pharmacy and Dialysis industries for quality control, product release and research. Our reagents are FDA licensed and can be used for testing in compliance with USP, EP and JP bacterial endotoxin test chapters, and our software is 21 CFR Part 11 Compliant.

ACC also operates a Contract Test Services (CTS) Laboratory which has specialized in testing for endotoxin and glucan contamination for over 35 years. Our CTS laboratory is GMP compliant, ISO registered and DEA licensed and is capable of handling all controlled drug substances except those included in Schedule 1. All testing services can be performed to FDA, USP, EP and/or JP regulatory guidelines. In addition to routine testing, our CTS Laboratory will customize endotoxin testing, troubleshoot difficult samples, develop and/or transfer LAL test methods, design and produce custom depyrogenation controls for oven validation and perform Low Endotoxin Recovery (LER) studies/protocols.

ACC also offers a clinical diagnostic product line and operates a CLIA-certified laboratory specializing in (1→3)-ß-D-glucans analysis to support the diagnosis of Invasive Fungal Disease (IFD).

Sustainability in Bacterial Endotoxin Testing (BET) – A Holistic Approach to Conservation and Recombinant Technology

Veronika S. Wills

Manager, Technical Services
Associates of Cape Cod, Inc.

***Veronika** has over 13 of years of experience in endotoxin testing, and currently manages the global technical team at ACC and is based at ACC's US Headquarters in East Falmouth, Massachusetts.*

Veronika is a subject matter expert when it comes to endotoxin testing and often provides expert sessions at global events focused on BET products and processes. Most recently Veronika has been speaking on the topic of recombinant technology as it relates to BET in the industry and abroad.

Veronika is a key contributor to ACC's sustainability initiatives and spokesperson on ACCs related projects, products and services.

Bacterial endotoxins can cause harmful symptoms, including fever and septic shock, if they find their way into a patient's bloodstream in sufficient concentrations. As a result, Bacterial Endotoxin Testing (BET) has become a fundamental safety requirement in the biopharma industry. Manufacturers must show that their finished products do not contain endotoxins exceeding the allowed limits.

The industry standard reagent for BET is Limulus Amebocyte Lysate (LAL), which is extracted from the white blood cells of the Atlantic horseshoe crab (Limulus polyphemus). For the past four decades, LAL reagents have been the only type of reagent approved by the US Food and Drug Administration (FDA) to test for bacterial endotoxins. In recent years, however, a new class of BET reagents have emerged: recombinant reagents.

Recombinant reagents are non-animal-based and produced using recombinant DNA technology – an attractive proposition for manufacturers looking to reduce their environmental footprint. Thanks to the fact that the recombinant reagents are non-animal-based, they may yield more reproducible and repeatable data. But do they perform as well as the industry standard LAL? That question is still being debated by the subject matter experts, though published studies show extremely promising data. As alternative reagents for testing of products per compendia, the recombinant reagent used has to be shown equivalent to LAL for each individual product tested. This presents some significant regulatory burdens currently associated with recombinant reagents.

First and foremost, the FDA does not license recombinant reagents and will not accept their use unless a compendial test has been performed showing that the reagent is equivalent to LAL. Crucially, this must be done by the individual end user in their own lab – a significant drain on resources. In addition, companies may struggle to understand exactly what the regulatory expectations are, especially given that local regulations and regulatory authorities in different jurisdictions have varying expectations of what they would like to see from the end user when validating an alternative reagent. The regulatory requirements for LAL reagents were harmonized over 20 years ago, but this isn't the case for recombinant reagents.

We are hopeful that these requirements will be harmonized in the coming years – and there are several groups working on this – but compendial testing remains a significant hurdle to the more widespread adoption of recombinant reagents as alternatives to traditional LAL reagents.

Making life as easy as possible

Given the substantial regulatory hurdles associated with implementing an LAL alternative, Associates of Cape Cod, Inc. (ACC) have set out to make things as easy as possible for the end user. ACC's PyroSmart NextGen™ recombinant Cascade Reagent (rCR) is the first and only reagent available on the market that mimics the LAL cascade – the reagent's mechanism of action – completely. This rCR is based on the genetic sequence of Limulus polyphemus and reacts with endotoxins in the same way as LAL. It launched in spring 2021 and is now commercially available globally.

The time to result with PyroSmart NextGen™ can be reproducibly achieved for the sensitivity of 0.005 EU/mL in 60 minutes (including preparation and test time), whereas traditional LAL reagents usually take 85 minutes or longer and rFC reagents (first generation recombinant reagents) take around 110 minutes – though this can be cut to 74 minutes by using a plate with predisposed CSEs. Unlike first generation rFC recombinant reagents, converting over to PyroSmart NextGen™ (rCR) does not require any changes to the user's current platform used for photometric LAL-based assays. The end user can use the same instruments and data analysis software as they do for traditional LAL; the only difference is the reagent. This really simplifies the process of demonstrating comparability with LAL. A considerable number of companies have joined ACC's evaluation program, which allows them to try the PyroSmart NextGen™ reagent and find out how suitable it is for testing their products while simultaneously collecting the comparability data required by regulators.

There is a lot of interest in alternatives to horseshoe crab-derived LAL reagents – especially as the industry as a whole has become more environmentally conscious over the past decade or so. But a combination of resources and internal knowhow limitations associated with proving comparability is a major hurdle that many end users simply cannot overcome – despite good intentions.

We are hopeful for greater regulatory harmonization to ease the burden on the end user but, until then, the process of adopting and proving comparability must be as straightforward as possible, and we are available to help with that process. We believe that allowing manufacturers to maintain their existing instrumentation and software platform will give more companies the option of choosing a non-animal-based BET reagent.

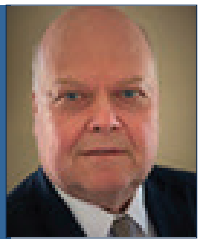
Created by and originally published in The Medicine Maker.

AN INTERVIEW WITH...

Brett Hoffmeister

LAL Production Manager
Associates of Cape Cod, Inc.

Chair to the Horseshoe Crab Advisory Panel
Atlantic States Marine Fisheries Commission



ASSOCIATES OF CAPE COD INCORPORATED

Specialists in Endotoxin and Glucan Detection

Can you give us a quick overview of your own background and also about Associates of Cape Cod, Inc. (ACC)?

Brett: Absolutely. Associates of Cape Cod has been located in Falmouth, Massachusetts since the beginning, since we were founded back in the '70s. The founder of our company, Stanley Watson commercialized LAL. We were the first to bring it to the market and we've been growing here since. It's been a great run for ACC. I started with the company in 2003. I've got a background in manufacturing. I love the ocean, I've been a resident of Cape Cod for longer than I care to admit, over 30 years. We found each other and I've been working with horseshoe crabs ever since. I'm really enjoying my experience here and the things I've learned.

Why is it important to test for endotoxins?

Brett: Endotoxins are everywhere. We're surrounded by them. They're a component of our life, but they're dangerous when they enter your bloodstream or spinal fluid. Endotoxins that you and I are exposed to on a daily basis, our bodies can handle, but if it does cross that bloodline, it becomes a problem. It's pyrogenic, it can cause a fever. It can cause terrible things to happen to the human body, up to and including death. Endotoxins are something that need to be tested for in anything that's injected or implanted into a human body.

Why are horseshoe crabs so important for endotoxin testing?

Brett: Horseshoe crabs have a component of their blood that clots around the presence of endotoxins. It's part of their immune system. Other animals have it but horseshoe crabs are readily accessible. It's a very primitive and simple mechanism to manipulate. They provide the LAL manufacturers in the United States, the raw material to produce the product that we make that test endotoxin. Essentially, we take those components from the blood, manipulate them and provide an assay in a test tube that's freeze-dried, it's stable and it's very accurate. It's very cost-effective.

Are there other ways to test for endotoxins as well?

Brett: There are. Prior to the '70s, the rabbit pyrogen test was the go-to test where pharmaceutical companies would inject a rabbit with, say, a sample flu vaccine for instance. They have to monitor the rabbit for two or three days to see if it spiked a fever. That test is still around today. It's not widely used, but it's a compendial test. It still exists. There's an MAT test that's based on human blood.

Most recently, there's a lot of news about RFC tests for Recombinant Factor C. Most recently ACCs actually released a new product, an rCR, a Recombinant Cascade Reagent, which more closely mimics the test based on the LAL test, so the same cascade. There are other tests out there, but the gold standard, the compendial test right now remains the LAL.

What does LAL stand for and what does it actually mean?

Brett: That's a great question. LAL stands for Limulus Amoebocyte Lysate. A lot of people say that crabs contain LAL and it's not the truth. The Limulus is based on the Limulus polyphemus, the American horseshoe crab. The lysate that we produce is based off of the amebocyte. We lyse the amebocytes and release those clotting agents that I was talking about. This is a product that we make from the blood of the horseshoe crabs.

If you're using horseshoe crabs for the testing, that must have an impact on the population, doesn't it, or does it not have any impact when you're using it for biomedical use?

Brett: That's a great question. There's a lot of debate about that. There's a lot of data, there's scientific data, volumes of scientific data that'll tell you that the impact is really minimal with what we do with the horseshoe crabs. In the United States, the primary use of horseshoe crabs is for bait. They use it to catch carnivorous snails or conch. The biomedical use is a much smaller component of that entire fishery. The population in the United States is healthy enough to withstand a bait harvest, and the biomedical mortality is a fraction of that bait harvest. It impacts a fraction of the population of horseshoe crabs that's measured in the tens of millions up and down the East Coast.

What do you say to people then who say that the population in the US is not doing well?

Brett: I would say, again, read the data. The Atlantic States Marine Fisheries manages the horseshoe crabs up and down the Atlantic East Coast, and individual states use those management directives to manage their own fisheries. States have the power to manipulate let's say, a quota. For instance, Massachusetts has a quota of 330,000 crabs that are allowed for bait. Massachusetts Division of Marine Fisheries cuts that down to about 165,000. I would let people know that there is a lot of people, a lot of scientists, a lot of biologists looking at the horseshoe crab fisheries on both the coast-wide level and the state level, and the populations are healthy.

In the Delaware Bay region alone, it's estimated that there's 30 million to 40 million adult crabs, countless juveniles, and it's probably safe to say that there's hundreds of millions of horseshoe crabs up and down the East Coast. The population itself is healthy. That said, horseshoe crabs are slow to mature. They are sexually mature at 10 to 12 years of age, so they're an animal that deserves and should be regarded as a fishery that needs to be managed. We need to monitor them, we need to look at them, and we want to minimize the impact that we do have.

Certainly, there are areas of concern. There are areas where population trends are not going the way we would like them to, and management addresses those through the process of fisheries management, again, on both the state and coast-wide level. I would encourage people to look at the data available with their individual states with the ASMFC and see the studies that are being done and see that there's a very healthy population out there.

What sort of conservation measures are being taken to help the horseshoe crabs?

Brett: Along those lines, again, the ASMFC regulates it. One of the things that they did in 2011 was to look at industry standards with the biomedical companies, and they put out a series of guidelines for the biomedical companies. They put in measures to reduce the bait harvest to a sustainable level. The goal of ASMFC is sustainable fisheries. Individual states can enforce two measures such as lunar closures like in Massachusetts and Rhode Island, where they protect the crabs during their most vulnerable time when they're up on the beaches spawning. There's no harvest allowed.

States implement things like size limits, bait bags that reduce the number of horseshoe crabs that are used by conch fishermen. There are areas that are off-limits to fishing. There are sanctuaries. There are national parks. There are areas that are off-limits to fisheries. There are conservation efforts along coast-wide levels. There are conservation efforts in place by the state and the manufacturers themselves. We work with some of the bay fishermen. In many cases, they self-regulate and look at the size crabs they have.

Certainly, ACC has taken an active part in conservation. Twenty or 30 years before fisheries were really managed, we had size limits. We had a return-to-sea policy with horseshoe crabs. Then most recently in the past four years, we've had an aquaculture project in place. It's our horseshoe crab sustainability project, where we're actually growing horseshoe crabs and releasing them to the wild.

Tell us more about that project. What have you been doing to ensure horseshoe crab sustainability?

Brett: This is a really neat project, and I'm happy to be part of it. We see a lot of horseshoe crabs come through our facility. In around 2017, we had the idea of, "What if we did some *in vitro* fertilization of eggs? We can harvest eggs from the crabs fairly naturally. We're not harming the crabs. We take a few grams out, we can stimulate, and get the gametes from males and females. We can fertilize them *in vitro*, and then we can hatch them out."

We've set up now a patent-pending system that we designed. The company was behind us financially, provided us with the resources that we needed, and we created this system that's very easy to increase and decrease the number of crabs and eggs that are available. Ultimately, what we do is feed the system eggs, hatch out the horseshoe crabs and grow them through their first and second instars till they're about the size of a pencil eraser, and then we can release them to the wild. We've got an aquaculture permit, a one of a kind from the DMF in Massachusetts. They give us three bodies of water to release. I'm happy to say that this year, we released our millionth crab to the wild, which is something that is worthy of celebration, I think.

Has COVID had an impact on the demand for LAL?

Brett: Another great question that we hear a lot, and honestly, no, it hasn't had a great impact. Certainly, COVID did impact the way we do business, a lot more stuff from home, and whatnot, but the demand for LAL does not increase exponentially. Despite the fact that the vaccines are in production, treatments for COVID are in production, one has to remember that the pharmaceutical industry has the ability to scale up significantly. Whether you're testing 1 liter, or 10,000 liters of water per se, the same amount of LAL can be used. That scalability is critical to the pharmaceutical industry being able to supply an increased number of vaccines, for instance, or IV solutions, for instance, but the demand for LAL can remain relatively the same.

If people want to know more about the different projects you talked more about and also about horseshoe crabs and about endotoxin in general, and the different testing processes out there. Where can they get more information?

Brett: Well, certainly, our website, www.acciusa.com, we've got a lot of information there about our sustainability practices, some of our products, including the new PyroSmart NextGen, which is our exciting recombinant product. Some of the data I talked about today is available with the Atlantic State's Marine Fisheries Commission, Massachusetts division of Marine fisheries, and there, you can look at population trends and learn a lot about horseshoe crabs.

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

James A. Akers,[1] John Duguid,[2] Deborah Gross,[3] David Hussong,[4] Karen McCullough,[5] Robert Mello,[6] Edward Tidswell,[7] Radhakrishna Tirumalai[8]

[1]*Akers Kennedy Associates, Inc., Leawood, KS*
[2]*Vericel Corporation, Cambridge, MA*
[3]*Merck & Co, West Point, PA*
[4]*Eagle Analytical, Houston, TX*
[5]*MMI Associates, LLC, Whitehouse Station, NJ*
[6]*Mello PharmAssociates, LLC., Reisterstown, MD*
[7]*Merck & Co, West Point, PA*
[8]*North Potomac, MD*

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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Novel Aptasensors for Endotoxin Detection Are Advancing Drug Discovery

Tim Sandle, PhD

Head of Microbiology and Sterility Assurance
Bio Products Laboratory Limited

Introduction

Assessing levels of endotoxin during the development of pharmaceutical products and for the assessment of patients under medical care forms an important part of the pharmaceutical and healthcare system. Depending on the product type, there are complexities involved. Bacterial endotoxin can form a stable interaction with other biomolecules thus making its removal difficult especially during the production of biopharmaceutical drugs. The detection of endotoxin (generally synonymous with lipopolysaccharide where the molecule's lipid A moiety possesses most of the biological activity) is important for patient safety due to its pyrogenic properties and ability to trigger a form of septic shock.[1] In addition, endotoxin can be difficult to detect when bound with protein in the human body. This makes endotoxin testing for certain applications significantly challenging, especially for drug development and medical research, such as screening patients with severe sepsis and septic shock.

To meet this challenge, innovations in endotoxin testing are being developed in the form of biosensor technology. The most promising of these is a form of electrochemical aptasensor[2] which detects endotoxin through voltammetric determination of lipopolysaccharide. Aptamers show great affinity toward their target analytes, such as with endotoxin. The aptamer recognizes the molecular target against which it was previously *in vitro* selected. There are several such sensors in research use and the development phases are promising. Furthermore, these technologies have the potential to meet the requirements of 'rapid microbiological methods' in that they meet the criteria of good performance, accuracy, repeatability and had a shorter time-to-results. This article reviews progress in this area of endotoxin detection.

Endotoxin Detection Sensors

Due to more established laboratory-based assays not being suitable for the detection of endotoxin bound with protein, research has been underway over the past decade to produce novel endotoxin detection methods and endotoxin sensors. Investigational methods include hydrophobic interactions, localized surface plasmon resonance, mass spectrometry, Surface Enhanced Raman Spectroscopy, optical methods, voltammetric methods, shear horizontal surface acoustic wave (SH-SAW) biosensors, and electrochemistry. The focus of this article is with those biosensor methods that are most applicable to pharmaceutical drug development. Biosensors are categorized into three groups of optical-, mass-, and electrochemical-based sensors. The electrochemical type is the most likely to be commercialized due to its relatively lower cost and for displaying acceptable performance characteristics including high reproducibility, sensitivity, and stability.

To date, trials involving biosensors have been attempted based on fluorescence, chemiluminescence, electrophoresis, and electrochemical techniques. Some methods recognize and quantify the entire endotoxin molecule, others detect part of the endotoxin molecule through a chemical reaction or signal, including binding to a specific ligand. The criteria for such methods is for the end-product to be low cost, easy to operate, to produce rapid analysis, to be capable of high sensitivity and selectivity. Succeeding with this is based on producing specific recognition mechanisms and sensitive signal transformations.[3] In particular, electrochemical biosensors have a long history as as rapid, robust, cost-effective and accurate analytical tools for the detection of various target molecules.[4] This concept is being extended to lipopolysaccharide.

Electrochemical Sensors

In order to develop suitable forms of endotoxin testing, an understanding of the intermolecular interaction of an endotoxin with other biomolecules is required. It is through this understanding that progress has been made with the development of aptasensors, which demonstrate how a modified electrode can have good selectivity for lipopolysaccharide over other biomolecules. The emphasis upon selectivity is important for a nontarget biological sample may cause a higher phase shift than the actual value, which would inevitably lead to incorrect experimental results being provided. Aptamers are oligonucleotide or peptide molecules that bind to a specific target molecule (such as single-stranded nucleic acids). They possess strong binding affinity and high specificity to various target substrates, such as ions, proteins and cells.[5]

Important criteria for these types of sensors are: Electrochemical activity, electrical conductivity, surface area, ease of functionalization and biocompatibility with the samples intended to be analyzed.

An electrochemical sensor based on dual functional copper (II) cation (Cu2+)-modified metal–organic framework nanoparticles for sensitive detection of bacterial lipopolysaccharide has been developed. In terms of how this type of sensor works, lipopolysaccharide is immobilized in gold nanoparticles and reduced graphene oxide by C18 alkane thiol chains. Graphene, which can be produced through chemical vapor deposition, is a 2-dimensional material with special physicochemical properties (including excellent conductivity and high mechanical strength). Graphene has good electrocatalytic activity toward small biomolecules, and it is biocompatible when used as a sensitive layer for the immobilization of biomolecules. This step is necessary given that lipopolysaccharide can interact with the C18 alkyl chains by strong intermolecular interactions.[6] Gold nanoparticles act as the signal amplification component, together with signal output components and molecular recognition components. Gold nanoparticles have a number of useful properties, including rapid electron transfer, high surface area, excellent biocompatibility and facile synthesis.[7] The main developmental point tends to center on the successful formation of each layer on the gold electrode when forming the nanocomposite.

Following the immobilization, Cu2+-modified metal–organic framework nanoparticles are captured by the anionic groups of the carbohydrate portions of liposaccharide molecules, and this functions as the recognition unit. This signal is accentuated by the Cu2+-modified metal–organic framework nanoparticles catalyzing dopamine oxidation, which generates aminochrome. This produces a strong electrochemical oxidation signal. Primary development issues include capturing dissociative lipopolysaccharide, which can be overcome with differential pulse voltammetry.

In a study conducted at East China Normal University,[8] an electrochemical sensor based on dual functional Cu2+-modified metal–organic framework nanoparticles was investigated by differential pulse voltammetry to monitor levels of lipopolysaccharide. The resultant method demonstrated a wide linear range from 0.0015 to 750 ng/mL, with an assigned limit of detection of 6.1×10^{-4} ng/mL (electrochemical impedance spectroscopy can be used to detect varying concentrations of endotoxin, before and after exposing to samples). This is demonstrated by assessing the linear relationship with the logarithmic values of the endotoxin concentrations, with a correlation coefficient of $R^2 > 0.98$ being desirable.

Assessing endotoxin in nanograms is important from the medical perspective, where leukocytes respond to lipopolysaccharide (at nanogram per milliliter concentrations with secretion of cytokines such as tumor necrosis factor-alpha (TNF-alpha), and the association where excess secretion of TNF-alpha causes endotoxic shock.[9] While the relationship between liposaccharide weight and potency is dependent upon the bacterial species, for standard control endotoxin *Escherichia coli* 055:B5, then 1 ng of endotoxin is approximately equivalent to 0.5 endotoxin units. There are also some complexities with types of endotoxin. The structures of lipid A vary, for example, between enteric and non-enteric Gram-negative pathogens and there is also sometimes heterogeneity within organisms as well as between differences between species.

Further trials used the sensor to detect LPS in mouse blood serum, in line with research into the connection between bacterial endotoxins (especially microbiome-derived lipopolysaccharide) and the inflammatory and pathological processes associated with amyloidosis and Alzheimer's disease.[10] With this evaluation, satisfactory results were achieved, including what was reported as good reproducibility, low detection limit, and specificity. Experiments have also demonstrated the recovery upon spiking lipopolysaccharide in clinical grade insulin, again demonstrating a promising application for the trace analysis of endotoxin in the field of pharmaceutical products.

In terms of advancing this form of biosensor, design obstacles that need to be addressed include overcoming the formation of insulating films that can arise through the interaction of the analytes with their probing molecules, which can become immobilized on the electrode surfaces. It is also important that the aptamer layer contains very few defects. There can also be problems with detection in relation to pH. The pH value affects the responses of biosensors to their analytes, especially under highly acidic or alkaline environments. Extremes of pH can damage aptamers or affect the interaction between aptamers and their targets. This is a problem that exists with the conventional *Limulus amebocyte lysate* (LAL) test. As with other enzymatic tests, LAL assay results are susceptible to changes in temperature and pH and to the presence of protease and/or impurities.[11] Different materials also interfere with the conventional LAL assay, such a nanoparticles (which represent an important area of medical application for drug delivery). In particular, gold nanoparticles are known to interfere with various *in vitro* assays like LAL due to their optical properties and potential for surface reactivity. The interference does not occur with the biosensor application.

Colorimetric and Fluorometric Sensors

Alternatives to the electrochemical sensors are those based on colorimetric and fluorometric technology. An example is 3-phenylthiophene-based water-soluble copolythiophenes (colored, aromatic solids) for the detection of lipopolysaccharide). Such

sensors display high selectivity to lipopolysaccharide in the presence of other negatively charged bioanalytes as well sensitivity with the detection limit at picomolar level.[12] Copolythiophene based sensors have bene shown to be capable of rapidly discriminating the Gram-negative bacteria (with lipopolysaccharide in the membrane) from Gram-positive bacteria (without lipopolysaccharide).

A new strategy from an alternative laboratory is based on the inhibition of ion transport by lipid bilayer derived from spontaneous assembly of lipopolysaccharides. With this colorimetric method, at acidic pH values, lipopolysaccharide binds with aminophenylboronic acid modified assembled magnetic nanospheres. This results in formation of lipid bilayer around the magnetic nanospheres. Under acidic condition, the lipid bilayer inhibits the release of iron ions from the magnetic nanospheres into the solution, which decreases the oxidized extent of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt mediated by hydrogen peroxide. This enables lipopolysaccharide to be detected over the wide linear detection range with the low detection limit. One application where the technology is being considered is in relation to water testing.[13]

Flow Cytometry-Based Magnetic Aptasensors

A related development is with flow cytometry based magnetic aptasensor assays for lipopolysaccharide detection. Such methods utilize two endotoxin-binding aptamers and magnetic beads to detect endotoxin. This is in the form of an endotoxin-conjugated sandwich complex fixed to magnetic beads, developed through the application of scanning confocal laser microscopy. Trials have shown that magnetic aptasensors can rapidly detect (in under one minute) endotoxin within a detection range of 10^{-8} to 100 mg/ml (including masking with bovine serum albumin, RNA, sucrose, and glucose, materials that can interfere with conventional endotoxin assays). These materials were selected because they are most likely to coexist with endotoxin in biological liquids.[14]

Lab-On-Chip Endotoxin Detection

Researchers have also succeeded in exploiting the optical features of nanoplasmonic transducers supporting Localized Surface Plasmon Resonances (LSPRs) for lipopolysaccharide detection. With this approach, ordered arrays of gold nano-prisms and nano-disks can be created through nanospheres lithography. The resultant transducers can be integrated into a simple and miniaturized lab-on-a-chip platforms and functionalized with specific antibodies as sensing elements for the detection of lipopolysaccharide. Such devices work via interactions of specific antibodies anchored on protein A-modified sensor chips. Due to the optical and physicochemical properties of plasmonic nanostructures, the test has a robust ability to concentrate light energy in nanoscale volumes, and subsequently the increased near field intensity in relation to incident light makes creates a useful transducing platform for endotoxin detection.[15] A good linear relationship between peak shifts and the lipopolysaccharide concentration has been demonstrated for the fabricated nano-structures with a detection limit down to 5 ng/mL. This means endotoxin detection is possible through integration with a proper microfluidic platform, which could be used to assess the endotoxin content of products under development. Moreover, this concept could also see microfluidic devices integrated into wearable medical devices, where such devices have suitable flexible properties.

Summary

Endotoxins are ubiquitous microbiological contaminants, and their role can pose problems for new drug development and in the clinical field, particularly when they cannot be accurately detected. Standard traditional techniques are not always suitable, and this has led to an evolving field of agile endotoxin detection systems. These are biosensor based endotoxin detection methods, several of which are moving towards commercially available detection methods. Much of the current work is centered around the stability of the methods.

In time, such developments may further influence of omics for endotoxin detection. Of the different sensors, the greatest success has been reported with endotoxin-detecting impedance aptasensors. This is provided that the main design and operability constraints can be overcome, which primarily relate to maximum aptamer probe coverage and pH.

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COVID-19's Impact on The Sustainability of the LAL Supply and Horseshoe Crab Population

Brett Hoffmeister
LAL Manufacturing Manager
Associates of Cape Cod, Inc.
East Falmouth, MA

Allen L. Burgenson
Lonza
Testing Solutions Global SME
Marketing-CDMB
Walkersville , MD

John Dubczak
Executive Director of Reagent Development and Pilot Plant Operations
Charles River Laboratories Charleston, SC

The COVID-19 virus has been impacting humans worldwide for nearly a year at this writing. Over this time there was a great deal of speculation regarding how the pandemic and vaccine production to combat the virus would affect the production of LAL, and possibly outstrip naturally sourced LAL availability. There was also speculation regarding the impact the potential increase in demand of LAL might have on American horseshoe crab populations. Multiple vaccines have been approved and millions of COVID-19 vaccines have been produced and are being utilized to inoculate human beings around the world. Not a single dose has been delayed because of insufficient LAL supplies to vaccine manufacturers. Vaccine production is underway with more expected to be released for use in the very near future. As representatives of LAL manufacturers, we wish to assuage concerns and provide assurance that the demands of LAL testing in support of COVID-19 vaccines doesn't adversely impacts its production, nor does it put product availability or the American horseshoe crab population at any increased risk.

Limulus Amebocyte Lysate (LAL) is a reagent produced from the white blood cells of the Atlantic horseshoe crab (*Limulus polyphemus*), which is utilized by pharmaceutical and medical device manufacturers in an FDA-mandated Bacterial Endotoxin Test (BET). Past and recent articles have given voice to concerns about the availability of the LAL supply to accommodate large scale vaccine testing. Other media suggest that single source-reliance on one species is an untenable risk with some have going so far as to suggest that LAL manufacturing jeopardizes the horseshoe crab population. The media attention has provided renewed opportunity for some to recirculate inaccurate and misleading information to the public about the impact of biomedical uses of horseshoe crabs and the status of the crab population in the US. It is important to have a discussion of the relevant factors involved. By reviewing some of the questions and issues recently expressed by the media and other platforms, we wish to educate the reader on the facts.

Is LAL-based product availability particularly fragile or easily interrupted by natural or manmade events?

No, the LAL industry has been manufacturing for over 40 years with no significant interruption of services resulting from hurricanes, floods, blizzards, oil spills, or other disasters. LAL manufacturers are geographically diverse, located along the East coast of the US: Associates of Cape Cod Inc. (Massachusetts), Charles River Laboratories (South Carolina), Lonza (Maryland) and Wako Chemicals (Virginia). This large geographical footprint helps avoid a natural or manmade disaster from interrupting product availability. LAL manufacturers all operate with contingency plans in place, and maintain inventory needed to meet customer demands. Millions of LAL

tests are performed annually. Production of COVD-19 vaccines is underway around the world and serves as a good example of the robustness of the LAL Bacterial Endotoxin Testing supply chain.

This is what we do.

Are horseshoe crabs endangered?

No. It is the duty of US Fish and Wildlife Service (FWS) to determine if an animal in the US is "threatened" or "endangered." The FWS has made no such claims to the status of the American horseshoe crab. They are not endangered; in fact, it's estimated that there are tens of millions of adult crabs in the Delaware Bay region alone.[1] In many areas, populations are growing considerably. However, in other parts of the world, horseshoe crabs are not so closely monitored. *Tachypleus tridentatus*, found in Southeast and East Asia, for instance, is used as food, fertilizer, and manufacturing for chitin and its LAL equivalent, TAL. In the US, American horseshoe crab harvest is regulated by state agencies and the Atlantic States Marine Fisheries Commission (ASMFC), which oversees the coast-wide fishery.

The ASMFC is made up of members from the US Fish and Wildlife Service, academia, fisheries managers, statisticians, scientists, and representatives of industry, government, and others who work to regulate horseshoe crab fisheries and monitor populations on the East Coast of the United States. Current management of the fishery is robust, and science based. The most recent benchmark stock assessment (2019) determined the overall number of American horseshoe crabs appears to be stable and is increasing in some areas.[1-3] It is reasonable to say that there may be more horseshoe crabs today than there have been for decades.

Does biomedical use of horseshoe crabs threaten the population?

The simple answer is no. The data show clearly that even a complete cessation of the biomedical fishery would have a minimal impact on the overall fishery mortality of horseshoe crabs. In fact, the population is so healthy that there is a coast-wide quota, to be lawfully harvested for bait, of nearly 1.6 million crabs. Actual landings based on market demand and state regulations are far less than that, at approximately 800,000 crabs annually. The biomedical mortality is roughly 10% of that of the bait industry.[1]

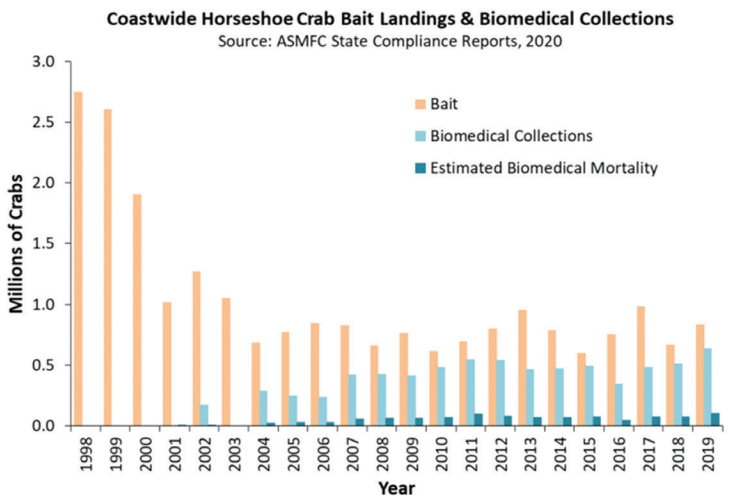
The 2019 stock assessment by the ASMFC states that the biomedical use of crabs has no impact on the population in the Delaware Bay region.[1] It is estimated that there are tens of millions of horseshoe crabs in the Delaware Bay region alone.[4]

It should be noted that in the areas where collection for LAL manufacturing exists, horseshoe crab populations are doing quite well and are stable and/or increasing. A recent study of nearly 175,000 crabs of which 68,000 were bled at LAL manufacturers over multiple seasons showed that long-term survival of those crabs, over multiple years, was as good or better than the survival rates of un-bled crabs.[1]

Will COVID-19 vaccine production threaten the population of HSC because of increased need of LAL?

No. The LAL test is an important quality control measure, required by law, for anything injected or implanted into the human body. It is already used millions of times annually on raw material, intermediates, and final products. It is important to note that a very small amount of LAL is needed to perform these tests. Modern pharmaceutical manufacturing has significant scalability throughout the industry. It takes roughly the same amount of LAL to test 1,000 doses as it does to test 100,000 doses. This serves as a reminder that even a high and unexpected demand for vaccines and medical products can be managed with the proper safeguards and planning that are in place.

The demands of BET testing materials worldwide can and are being absorbed with available inventory and without significant negative impact on the pharmaceutical industry or supply chain. This ability to scale up production of pharmaceuticals alleviates any sudden and unexpected increase in testing demands and need for significant increases in LAL inventory. There is no



* Biomedical collection numbers, which are annually reported to the Commission, include all horseshoe crabs brought to bleeding facilities except those that were harvested as bait and counted against state quotas.
* Most biomedical crabs collected are returned to the water after bleeding; a 15% mortality rate is assumed for all bled crabs that are released. This number plus observed mortality reported annually by bleeding facilities via state compliance reports is noted in the above graph as 'Estimated Biomedical Mortality.'

Figure 1.

significant increase in the use of horseshoe crabs and no threat to the population because of COVID-19 vaccine production.

What are the threats horseshoe crabs face today?

According to the 2019 ASMFC Stock Assessment report, the following are the major sources of horseshoe crab mortality:

- Bait Harvesting
- Bycatch from other fisheries
- Loss of habitat due to erosion prevention measures (riprap, seawalls, etc.), and human encroachment on spawning grounds
- Stranding after spawning (estimated 10% mortality of entire Delaware Bay population, annually)

Like any sea creature, horseshoe crabs are dependent on a suitable environment where they can live and reproduce. Water quality is an important factor as is having suitable beaches in which to lay their eggs. Fertilizers, septic systems, and other forms of pollution can greatly reduce the quality of water the crabs depend on. Sea walls, rip-rap, and jetties can manipulate the natural movement of sand on beaches and affect spawning habitats. Beach nourishment, the practice of bringing in truckloads of sand to beaches to replenish what's lost, or make them look nice, can bury millions of eggs before they hatch if not carefully planned. We all have a part in protecting this valuable resource.

Is there any oversight of the manufacturing and collection processes?

Yes, LAL manufacturing is a highly regulated/audited and complex process that provides a critical lifesaving assay for the pharmaceutical and medical device industry. Manufacturers are regulated by the Food and Drug Administration (FDA) and must comply with strict regulatory standards to certify product quality, efficacy, and safety. In addition, routine audits of the process are conducted by the FDA, the International Organization for Standardization (ISO), fisheries managers, and customers. Fishers collecting crabs for LAL manufacturers are mandated to follow local regulations as a condition of permitting. In 2011, the ASMFC partnered with LAL manufacturers, citizens groups, fishers, and dealers to document industry best management practices (BMP). Many of these practices, such as a swift return to the water and careful handling practices, have been in place by manufacturers for over 40 years, and help to ensure quality product while minimizing the impact on the individual crabs. This in turn helps ensure survivability of the animal and the population. In most East Coast states there are regulations in place that help to protect the HSC populations.

What do LAL manufacturers do to support conservation?

LAL manufacturers have practiced conservation measures since the beginning of this process, long before regulatory bodies began managing the fishery. In addition to the decades-long catch and release policy, LAL manufacturers work closely with fisheries managers and have members on the advisory panel of the ASMFC. They have helped or initiated conservation measures such as closing areas to bait fishing, participating the "rent a crab" program, which utilizes crabs from the bait industry, and supporting quotas and size limits. They support and have initiated aquaculture of HSC for release to the wild. LAL manufacturers financially support organizations such as The Ecological Research & Development Group (ERDG), aquariums, and the Virginia Tech trawl survey. Volunteers participate in spawning surveys, tagging studies, and the "Just Flip Em" campaign, which saves thousands of crabs each year. Many employees also routinely work with universities, schools, and citizen groups helping to increase public awareness and educate people about these remarkable animals.

Conclusion

The proposition that populations of horseshoe crab are declining because of their use in biomedical testing ignores the fact that there is a healthy and stable population of crabs in the US and that the impact of the LAL industry is minimal. The Review Panel consisting of representatives from academia, the National Marine Fisheries Service, and the Maine Department of Marine Resources, agreed with the ASMFC assessment team's approach, but noted:

> *…some covariates such as season of harvest, size/condition of crabs, and location that are worth investigating. However, additional data and analyses are not likely to significantly alter assessment results due to the modest magnitude of biomedical mortality. As such, while an uncertainty, the biomedical mortality rate should receive less focus in future assessments.*

In conclusion it is reasonable to state that the horseshoe crab population in the US is viable and healthy, the biomedical industry does not impact this population negatively, and the supply of LAL is also robust and healthy. Alarmists who suggest otherwise do so by ignoring the scientific facts and without any true knowledge of the LAL industry, the horseshoe crab fishery, or population data.

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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:

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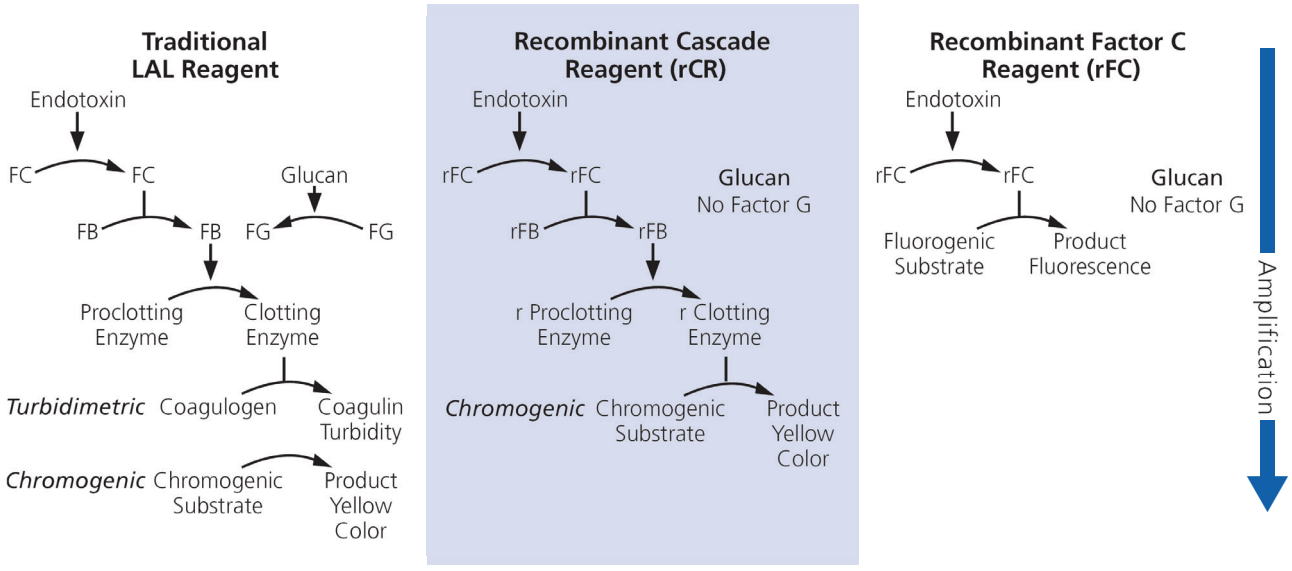
- No Cross Reactivity With 1,3-β-D-glucans
- Same Instrument
- Same Preparation Steps
- Meets Your Sustainability Objectives

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

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Validation Strategies for Gel-Clot Bacterial Endotoxin Testing

Crystal M. Booth, M.M.
Regional Manager
PSC Biotech

Introduction

Bacterial endotoxin testing (BET) utilizing *Limulus Amebocyte Lysate* (LAL) was introduced in the 1970s (The Horseshoe Crab, 2013). By 1980, the Food and Drug Administration (FDA) had developed a draft guideline and procedures for the test. In addition, the United States Pharmacopeia (USP) had developed Chapter <85> Bacterial Endotoxins Test making the LAL test a compendial assay (The Horseshoe Crab, 2013).

The gel-clot assay was one of the first bacterial endotoxin methods introduced to the industry. It established calculations and techniques that are important in properly understanding the assay. Even after 40 years of innovation, principles established with gel-clot testing are important to retain. This article briefly revisits the gel-clot bacterial endotoxin testing concepts required to validate the assay with accuracy and precision.

Gel-Clot Testing Overview

Since the 1980s, newer methods and guidelines for the bacterial endotoxin tests have been developed. As newer methods are developed and accepted, the core fundamentals of the LAL test can be lost in the automated technology process. The loss in technique and understanding of bacterial endotoxin testing could lead to confusion when analyzing the results.

Companies may perform the gel-clot assay for many reasons. These reasons could include the costs of the equipment for the modern assays, or even that the gel-clot assay is required per the compendia. USP <85> states that "in the event of doubt or dispute, the final decision is made based upon the gel-clot limit test unless otherwise indicated in the monograph for the product being tested (USP <85>)."

Gel-clot testing is a manual process. To perform the assay, an endotoxin standard series is made with control standard endotoxin (CSE), LAL reagent water (LRW), and LAL. Lambda (λ) is the sensitivity of the LAL being used for the test. It is also printed on the certificate of analysis (CoA) and on the vial of LAL. The dilutions of the standard curve that are tested is 2λ, λ, ½λ, and ¼λ. The standard curve must be positive within a 2-fold dilution of λ to be valid.

The product dilution that is being analyzed is tested in equal parts with the LAL reagent. Typically, this is 100 microliters (µL) of the product dilution and 100 µL of LAL mixed. Positive product controls are also prepared by making a dilution of the product inoculated to a 2λ level of endotoxin. Negative controls of the uninoculated diluents (e.g., LRW) are also analyzed.

All test tubes are incubated at 37°C. At the completion of a 1-hour incubation plus or minus 2 minutes, the tubes are inverted 180° to look for a solid clot. Anything other than a solid clot is considered a negative result. While performing gel-clot testing, care must be taken to not jar the test tubes because the clot could be knocked loose.

BET Calculations

There are several calculations that are important in endotoxin testing. One of these calculations is the endotoxin limit (EL) calculation. When setting specifications for a product, it

Crystal M. Booth, M.M., *is a Regional Manager a PSC Biotech and has over 20 years of experience in pharmaceutical microbiology. She obtained her master's degree in microbiology from North Carolina State University. Crystal is a seasoned award-winning technical writer and author of Method Development and Validation for the Pharmaceutical Microbiologist. During her career, Crystal has worked in microbiology, consulting, quality assurance, CDMOs, R&D, and quality control laboratories. Crystal has developed and validated numerous microbial methods and has worked with many different product types.*

is wise to consult the compendial monographs first. If a product will be available in many countries, use the most stringent monograph EL as the specification to be compliant in various regions of the world. If a monograph is not available, the EL calculation described in USP <85> may be utilized to calculate the specification.

The EL calculation is used to determine if endotoxin levels in product are safe. It is calculated by using the formula EL=K/M where "K" is a threshold pyrogenic dose of endotoxin per kilogram (kg) of body weight, and "M" is equal to the maximum recommended bolus dose of product per kg of body weight. Typically, "K" is set to 5 endotoxin units (EU)/kg unless intrathecal or radioactive drugs are to be tested (USP <85>).

When the product is to be injected at frequent intervals or infused continuously, "M" is the maximum total dose administered in a single hour period (USP <85>). "M" in the United States is typically considered 70 kg for the average human body weight and 60 kg is usually used in Japan when the calculation is performed.

In addition to knowing the EL established by the calculation, some companies may add on a safety factor to their endotoxin limit. For example, a company may calculate that the limit must be not more than 1.5 EU/mL of product. To be safe, the company may choose to utilize 0.3 EU/mL as their EL to ensure the endotoxin levels in the product do not come close to the maximum allowable limit. When establishing limits, it is important to consider all contributing sources of endotoxin such as water, components, and raw materials (Charles River Endosafe Workshop, 2008). The maximum allowable amount of endotoxin a 70 kg human can be exposed to in an hour is 350 endotoxin units.

There are two calculations that are important in understanding how much a product can be diluted and generate valid results. The first calculation of this type is the maximum valid dilution (MVD). To find the MVD, multiply the endotoxin limit by the sample concentration and divide that result by lambda (USP <85>).

$$MVD = \frac{\text{Endotoxin Limit X Sample Concentration}}{\lambda\ (EU/mL)}$$

The next calculation comes in handy when endotoxin toxin limits are expressed in milligrams. This calculation is known as the minimum valid concentration (MVC). To find this value, divide lambda by the endotoxin limit (Charles River Endosafe Workshop, 2008).

$$MVC = \frac{\lambda}{\text{Endotoxin Limit}}$$

The geometric mean is another calculation utilized in endotoxin testing. It is explained more in USP <85>. This calculation is typically used when qualifying a new analyst or performing quality control on new lots of reagents.

Two additional calculations are for finding the results of the assay. In the first calculation, divide lambda by the test concentration. This gives the result of the amount of endotoxin units that are present in milligrams (mg) of product.

$$\frac{\lambda}{\text{Test Sample Concentration}} = EU/mg$$

If both tubes are negative, the result will be reported as less than (<) the calculated value (e.g., <2.5 EU/mg).

The next calculation is for results that are expressed in volume. Simply multiply lambda by the dilution factor. For example, 0.125 EU/mL times 10 for the dilution factor is 1.25 EU/mL.

$$\lambda \text{ X Dilution Factor} = EU/mL$$

The last calculation is useful in finding the dilutions in the standard series for the assay. The calculation can be used to easily find any desired value, such as the 20λ value from the reconstituted CSE vial. It is the known endotoxin value divided by the desired endotoxin value (Charles River Endosafe Workshop, 2008). For example, a vial contains 50 EU/mL and lambda is 0.125 EU/mL. The desired value is 20λ or 2.5 EU/mL. The result 20. This means that a one to twenty (1:20) dilution should be made to get 2.5 EU/mL.

$$\frac{\text{Known Endotoxin Value}}{\text{Desired Endotoxin Value}} = \text{Required Dilution to reach Desired Endotoxin Value}$$

Twenty lambda (20λ) can be used as a hot spike to make positive controls. Make a hot spike by adding 10 µL of 20λ CSE to 100 µL of the product dilution. This creates a 2λ endotoxin spike that must come out positive to be valid.

Twenty lambda (20λ) can also be used to easily prepare the endotoxin standard series. A 1:10 dilution can be made from the 20λ dilution to achieve the 2λ dilution. Following the preparation of the 2λ dilution, a series of 1:2 dilutions can be prepared from the 2λ dilution for the remaining dilutions of the standard series.

Method Development Considerations

The first step in any method development process is to get to know and understand the product. Work closely with the development scientists to learn product traits such as solubility, potential interference issues, endotoxin limits, dilutions, and the pH.

Perform any trial-and-error testing in a method development notebook. Products need to be screened for any interference issues and a method needs to be established to overcome the issues if they are present. Performing this work in a method development notebook allows for a clear and concise validation protocol package to be created. Once a method is identified, it can be executed for validation neatly in an approved protocol.

The interference (inhibition or enhancement) screen is usually performed by diluting down to the MVD or MVC in serial dilutions, such as 1:2 dilutions. Inhibition occurs when positive controls that should be positive, give negative results or when the endotoxin amount of the dilution cannot be determined. Enhancement occurs when a positive control is a lot higher than it should be or negative products are giving positive results.

In gel-clot testing enhancement is difficult to detect. It must be assumed that the product is giving a positive result unless it can be proven otherwise. The most common ways to get over interference issues is by dilution, balancing a pH if needed, or using an endotoxin specific buffer to eliminate possible Glucan interference. When performing endotoxin testing, the pH of the product dilution and the LAL reagent combined must be between a pH of 6-8 for the assay to work properly (Charles River Endosafe Workshop, 2008).

Method Validation Strategies

Once a method has been developed, clearly describe it in a method validation protocol. The protocol should describe performing an inhibition and enhancement screen once to show that the method of choice works in overcoming any interference issues. This is important for new or tricky products that may be known to interfere with the BET assay.

The protocol should describe which dilution that worked from the method development phase or interference screen. Choosing the 3rd highest positive dilution in the interference screen is a good choice (Charles River Endosafe Workshop, 2008). It is not the only choice, but it is typically a good choice.

During the validation stage, three lots of product are usually used for commercial product method validations. If a product is a clinical product, one lot performed three times may be sufficient until more products become available, and the formulation is finalized.

Recall that the pH of the LAL reagent and the product dilution mixed must be documented to be between 6 and 8. If the pH needs to be adjusted during the validation phase, it will need to be adjust the same way during routine testing (Charles River Endosafe Workshop, 2008).

To perform the method validation, a product standard series is made in addition to the endotoxin standard series that is prepared in LRW. To prepare a product standard series, the production dilution to be tested is prepared. This dilution is inoculated with enough endotoxin to be equivalent to the 2λ endotoxin value. This inoculated 2λ product dilution is then diluted to the λ, ½ λ, and ¼ λ by performing 1:2 serial dilutions in the uninoculated product dilution. Each one of these dilutions of endotoxin inoculated product is tested in quadruplicate. A negative product control (NPC) is also performed in quadruplicate. The NPC is the uninoculated product dilution.

In addition to the product standard series, two replicates are typically analyzed for the standard series of the endotoxin prepared in LRW. Figure 1 demonstrates the setup of test tubes for the product standard series and the standard series in LRW.

Like the endotoxin standard series, the product standard series must be positive within a 2-fold dilution of lambda to be valid.

During the bacterial endotoxin assay, instances of invalid results may arise. Invalid assays are different from out of specification results. However, they may require an investigation to find the cause of the invalidity. Some common causes include, pipetting errors, analyst error, subpotent endotoxin standards, contamination, or dilution errors.

Conclusion

This article reviewed some of the validation strategies and core concepts for gel-clot BET. The gel-clot method is a manual test that utilizes equations and aseptic laboratory testing techniques. Newer methods and guidelines have been developed for endotoxin testing over the years. However, the core fundamentals of the LAL test can be lost when newer technology is adopted if employees are not properly trained.

According to USP <85>, the gel-clot method is required to be used "in the event of doubt or dispute with other endotoxin testing methods, unless otherwise indicated in the monograph for the product being tested (USP <85>)." Some companies may also choose to perform the gel-clot method due to the price of the assays using the modern methods. Knowing the fundamental concepts established from the gel-clot method and the validation can help analysts troubleshoot, analyze results, and completely understand the assay. Having the base concepts of bacterial endotoxin testing and validation strategies are essential for testing with accuracy, precision, and confidence.

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Figure 1. Standard Series Dilutions

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