

# ENDOSAFETIMES

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## PERSPECTIVE

James F. Cooper, Ph.D., Consultant and Founder of Endosafe, Inc.

Early development of the LAL test and the role of regulatory and Pharmacopeial organizations are discussed in the second of a two-part history series by Marlys Weary in this issue of the *Endosafe Times*. The endotoxins test exemplifies how scientists and regulators have interacted successfully to realize the full potential of a new diagnostic tool. In her article, Ms. Weary has identified significant benchmarks leading to acceptance of the Bacterial Endotoxins Test (BET).

Fredrick Bang made the critical scientific observation that led to this discovery. I had the good fortune to hear Fred speak on serendipity in science just before his untimely death in 1981. His studies on marine-invertebrate response to injection of bacteria arose out of his interest in immunity and from his speculation that ancient species might reveal primitive

immunological functions. During the summers of 1950 and 1951, Bang injected various bacteria into the circulatory system of horseshoe crabs with the expectation of generating agglutinins. Rather, injection of a marine pathogen, *Vibrio*, produced an infection and subsequent death by intravascular coagulation. He also observed that Gram-negative bacteria (GNB) and a heat-stable extract of these bacteria also produced coagulation; Gram-positive bacteria did not produce this response.

Upon discussing his finding with colleagues at the Johns Hopkins University (JHU), Dr. Conley suggested collaboration with Jack Levin, a Fellow in Hematology. Levin was studying the Schwartzman reaction, a mammalian clotting phenomenon associated with GNB. During the 1960s, Levin and Bang found that the coagulation system was

contained in the amebocytes and that release of the coagulation factors enabled Limulus plasma to be gelled by bacterial endotoxin. Levin began to use the time required for formation of a firm gel in a 10x75 mm test tube to estimate the concentration of endotoxin.

In 1969, I met Levin and Bang while studying Radiological Sciences at The JHU. I suggested a collaboration to compare the sensitivity of the new *in vitro* endotoxins test with the official Pyrogen Test. My interest stemmed from the need to find a rapid endotoxins test for the new radiopharmaceuticals that we were developing at that time. A comparison study revealed the remarkable sensitivity and specificity of the LAL for endotoxin and demonstrated the feasibility of a screening test for injectable drugs.

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## A Short History of the Limulus Amebocyte Lysate (LAL) Test

Marlys Weary, MERIT Consulting Services

In the last issue of the *Endosafe Times*, we followed the history of pyrogen testing from its inception to the 1942 inclusion of the rabbit pyrogen test into the 12<sup>th</sup> edition of the United States Pharmacopeia (USP).<sup>1</sup> From that time forward, the USP <151> Pyrogen Test, in general, served the pharmaceutical and medical device industries very well in providing a test method to assure the absence of pyrogens from intravenously injected medications. However, since the rabbit <151> Pyrogen Test has always been a time-consuming, elaborate and expensive test to perform, the pharmaceutical industry was always on the lookout for a better, more reasonable alternative test method. The best answer to date has been the Limulus Amebocyte Lysate test, commonly referred to as the LAL test.

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## A Short History of the Limulus Amebocyte Lysate (LAL) Test

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The LAL test is an *in vitro* pyrogen test method that is specific for the detection of Gram-negative bacterial endotoxin pyrogen. For this reason, it is referred to by the USP as the <85> Bacterial Endotoxins Test, or BET for short. When the LAL test was originally introduced, concerns were expressed that its specificity would limit its application. However, experience gained over the many years of its use has proven that bacterial endotoxin pyrogen is the primary pyrogen of interest to pharmaceutical and medical device manufacturers, and for this reason, the LAL test has steadily gained acceptance around the world as a replacement for the rabbit pyrogen test.

The LAL test employs a diagnostic reagent for the detection of bacterial endotoxin that is prepared by lysing amebocyte blood cells from the Atlantic horseshoe crab, *Limulus polyphemus*; hence the reagent's name, Limulus Amebocyte Lysate. The ability of Limulus blood cells to form a gelatinous clot in the presence of foreign substances had been reported as early as 1885 by Howell<sup>2</sup> and again at the beginning of the 20th century by Loeb.<sup>3,4</sup> However, the origins of the LAL test are usually attributed to the work of Bang, who was the first to recognize the association between bacterial endotoxin and Limulus blood coagulation.<sup>5</sup>

Dr. Frederick B. Bang was a Pathobiologist at the John Hopkins University School of Medicine who spent his summers performing research at the Marine Biological Laboratory in Woods Hole, MA. His primary academic interest was infectious diseases, but since the Marine Biological Laboratory required

its researchers to study marine organisms, Bang opted to work with horseshoe crabs. He made this selection after viewing a dying horseshoe crab that had been previously injected with seawater. When Bang cultured a Gram-negative *Vibrio* bacterium from the seawater, and re-injected it into other healthy Limuli, both the living bacteria, or its extracted endotoxin, caused the animals to die, not from infectious processes, but from intravascular coagulation.<sup>6</sup> After publishing these observations, Bang's investigations of this oddity were put aside until the summer of 1963. At that time, Dr. Jack Levin, a young Hematologist who had an interest in the effects of endotoxin on platelets and human blood coagulation, joined Bang at Woods Hole for a summer of research on endotoxin-initiated horseshoe crab blood coagulation. Subsequently, this collaboration resulted in the publication of several scientific papers that explained a simple mechanism of endotoxin-LAL interaction<sup>7,8,9</sup> followed by a description of a semi-quantitative LAL test method for the presence of endotoxin in blood.<sup>10</sup>

Although Levin's original interest in the LAL test was for its potential as a clinical assay for diagnosing endotoxemia in patients, the test has found its most celebrated application as an industrial pyrogen test. The use of the test to detect endotoxin in pharmaceuticals was first described by Cooper in 1971, who demonstrated that the Limulus test was approximately 10 times as sensitive to endotoxin as the official rabbit pyrogen test when both tests were used to evaluate naturally pyrogenic short-lived radiopharmaceutical agents and prepared solutions of two bacterial endotoxins.<sup>11</sup>

### LAL POINTERS

#### The Myth of Plastic Adsorption of Endotoxin

During the 1980s, BET labs became interested in using disposable plastic materials as a convenient and less expensive alternative to glass. It became readily apparent that there was a problem with plastics. Two publications appeared in 1986 in the PDA Journal that speculated inaccurately about adsorption of endotoxin to plastic. First, a multi-center study on recovery of LPS observed poor recovery from medical devices without using drastic measures, such as extraction with a surfactant and sonification. Secondly, an LAL supplier published that poor recovery was due to adsorption to plastics. Subsequent experiments found that the inhibition was due to organic materials leached from container surfaces and that the problem was associated with poor dispersion of LPS (RSE and CSE preparations) and not environmental endotoxin. As a result, many assumed that plastic bound and harbored endotoxin. The FDA tightened the tolerance limit for endotoxin extracted from devices in comparison with drugs. The reference cited in the Laboratory Notebook section reviews the recovery issues and confirms that validation of endotoxin recovery from plastic devices has no merit.





The following year, the FDA Bureau of Radiological Health and the NIH Division of Standards published the results of studies in which 155 radiopharmaceuticals and biological products were pyrogen-tested by both the rabbit test and the LAL test. They concluded that the LAL test was “a rapid, sensitive and reproducible method for detecting pyrogen in these products.”<sup>12</sup> Shortly thereafter, LAL reagents became commercially available, and the pharmaceutical and medical device industries began investigating the possibility of using the LAL test as an alternative pyrogen test method for the USP rabbit <151> Pyrogen Test.

In 1973, the *Federal Register* announced the intention of the FDA to license LAL reagent as a biological product. That announcement also proclaimed the usefulness of the reagent for detecting endotoxin.<sup>13</sup> Then in 1977, another *Federal Register* announcement gave conditional approval for the LAL test as a final release test for medical devices and biological products, provided that product manufacturers submitted appropriate test-validation data to amend their product’s registration documents or license.<sup>14</sup> For a period of time, each individual FDA agency set its own policy for using LAL for product endotoxin testing. The Bureau of Medical Devices even established its own unique endotoxin standard, after using that standard to support industry collaborative studies to validate appropriate endotoxin limits for the device products that it regulated.<sup>15</sup> However, as the use of the LAL test

became more and more prevalent, the FDA decided that a single standardized document was needed to govern all FDA-regulated products that were subject to LAL-testing. An FDA task force was formed by representatives from the Center for Drug Evaluation and Research, the Center for Biological Evaluation and Research (CBER), the Center for Devices and Radiologic Health, and the Center for Veterinary Medicine. Their joint effort to standardize the FDA LAL test-validation criteria was published in final form in December 1987.<sup>16</sup>

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### The origins of the LAL test are usually attributed to Frederick Bang

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This guideline set forth acceptable conditions for the use of the LAL test as a replacement for the rabbit pyrogen test for the products regulated by all four FDA agencies. Although the guideline is not a legal requirement, the document explains that those who follow the test-validation procedures outlined in the guideline may be assured that these procedures will be acceptable to the FDA.

Meanwhile, the USP first published the <85> Bacterial Endotoxins Test as an informational chapter in 1980, in *USP XX*. However, the new test was not proposed as an alternate to the rabbit <151> Pyrogen Test at that time, since the compendium was not yet ready to assign endotoxin limits to the various USP articles included in its monographs. The first USP article monographs to have their <151> Pyrogen Test requirements replaced with the BET test were those for 29 radiopharmaceuticals and five pharmaceutical waters. This occurred in November 1983, in an Addendum to Supplement 4 of *USP XX*. Later, in 1987, the USP subcommittee responsible for revising the general chapters of the compendium, announced its intent to replace the <151> Pyrogen Test with the BET for all USP articles for which the BET could be validated.<sup>17</sup> The first large-scale conversion of the <151> Pyrogen Test to the BET occurred in Supplement 5 to *USP XXII*, which was released in September 1991. At that time, the BET became the official endotoxin test for 185 USP articles. By the time Supplement 8 of *USP XXIII* had become official on May 15, 1993, the BET was an official compendial test requirement for more than 480 USP articles and for more than 650 articles by 2001.



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is being used to  
conduct biological  
research in space

As stated in <1196> Pharmacopeial Harmonization, the BET was successfully harmonized on an international basis by the Pharmacopeial Discussion Group (PDG), which consists of representatives from the European Directorate for the Quality of Medicines in the Council of Europe (EP), the United States Pharmacopeial Convention, Inc. (USP), and the Japanese Pharmacopoeia in the Ministry of Health, Labor and Welfare (JP). Final agreement on the harmonized document for the LAL gel-clot method as well as the two photometric methods, turbidimetric and chromogenic substrate techniques, was completed in September of 1999, with the gel-clot method considered a referee test in case of conflict. The results were published thereafter in each member's respective pharmacopeial forum document, and the harmonized USP test procedure was implemented in the Second Supplement of *USP 24* on January 1, 2001.<sup>18</sup> Per general chapter <1196>, the USP Endotoxin Reference Standard for the BET is harmonized with the International Reference Standard for endotoxin and the EP Endotoxin Reference Standard and indirectly harmonized with the JP Endotoxin Standard that is indexed to the International Standard. The result is that 1 USP Endotoxin Unit = 1 International Endotoxin Unit = 1 EP Endotoxin Unit.

This just about brings this short history of the LAL test up to date. One can only speculate on what the future brings for the test. However, with Charles River's January 4, 2007 press release, announcing that its portable endotoxin testing system, the Endosafe® - PTS™, successfully reached the International Space Station as part of NASA's ongoing efforts to conduct biological research in space, we would have to say that "the sky's the limit!"

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# LABORATORY NOTEBOOK

## Accessories for Endotoxin Testing

### Overview

The generation of credible BET (Bacterial Endotoxins Test) results necessitates robust test procedures, well-maintained equipment and properly trained personnel.

A critical part of robustness for endotoxin testing is the choice of accessories that enables data collection free of artifacts and sources of interference. Nothing is more devastating to lab management than the frequent occurrence of invalid tests that must be explained and redone. The most expensive LAL test is the one that must be repeated because of invalidity. The selection of non-interfering accessories for a BET is not only a pharmacopeial directive but also a regulatory expectation. This discussion is the first of a two-part series that addresses interference from accessories. This part focuses on issues that lead to robustness in endotoxin testing when considering the selection of plasticware. The second part will consider water and glass accessories and suggest a scheme for managing such problems.

The root causes of accessory interference may be traced to influences on LAL Reagent quality or Control Standard Endotoxin (CSE) potency. Activation of LAL Reagent may arise from touch contamination by operators and contact with accessories that contain trace amounts of endotoxin; examples of the latter will be discussed. Subtle effects may be seen, such as increased background noise in kinetic BET studies or reduced recovery of positive controls. Of course, there can be obvious manifestations, such as gels in the negative controls for gel-clot assays.

A CSE solution is the component in endotoxin testing that may be most at risk to artifacts and interference. The principal reason for CSE fragility is the low concentrations inherent in standard solutions, which are in the range of parts

per billion or trillion in working standards. That makes endotoxin susceptible to sources of soluble impurities, notably, extractables from glass and plastic containers. With time, endotoxin standards seem to disappear due to poorly understood aggregation phenomena. Good accessories and diligent vortex mixing are needed to maintain standard potency. The most common problem is loss of CSE potency in low-concentration standards for kinetic studies. Since the standard curve is an inverse relationship with reaction time and endotoxin concentration, a weak standard series may result in over-reporting of analysis results and enhancement of the positive controls.

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### Proper selection of accessories is critical in LAL testing

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It is important to interject at this point that the instability in potency described above is a property only of purified endotoxins, such as the lipopolysaccharide (LPS) in CSE reagents (usually derived from *E. coli*). In contrast, environmental (naturally occurring) endotoxin is remarkably stable and dispersible in aqueous solutions. Purification of endotoxin removes proteinaceous components that render LPS poorly dispersible and less stable in potency than endotoxin and leads to increased molecular aggregation, loss of LAL activity and lower toxicity in mammals. If we only worked with environmental endotoxin, not LPS, no vortex mixer would be needed in the BET lab, because it often retains its potency in simple solutions for years.



### Plastic supplies

The general absence of water exposure during fabrication and the heat of extrusion processes for plastic materials should render these items at very low, if any, risk for endotoxin contamination. However, plastic materials may be the most problematic accessories in the BET lab. The USP warns: "if employing plastic apparatus such as microplates and pipette tips for automatic pipettors, use only that which has been shown to be free of detectable endotoxin and not to interfere with the test."

Disposable plastic **pipettes** are notorious for contributing contaminants that at least partially activate LAL reagent. For example, the use of these items for LAL rehydration, sample dilution and CSE solutions often causes low-level contamination in kinetic LAL studies. Depyrogenated glass pipettes are the best choice for LAL dispensing.

Cotton and other cellulosic materials are particularly problematic for LAL applications. A cotton boll is exposed to environmental bacteria and fungi during agricultural growth; it becomes highly contaminated with endotoxin. Further, cotton is the purest form of cellulose and contains substantial amounts of LAL Reactive Glucans (LRG). Therefore, pipettes that contain protective cotton or cellulosic plugs should be banned from the BET lab.

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Polypropylene **pipette tips** are universally used to transfer LAL reagent and other test components for LAL testing. To our knowledge, there is no recent report of interference or contamination assignable to polypropylene pipette tips for LAL dispensing. Skepticism about the meaning of “pyrogen-free” for commercial sterile pipette tips is justifiable. Suppliers may simply extract a small number of tips and test the extraction to report that the solution is  $<0.5$  EU/mL. Such low level of sampling is hardly sufficient evidence for a label of pyrogen free. However, one supplier certifies a level of  $<0.001$  EU/mL for tips, far more stringent than needed. Generally, bulk packages of sterile pipette tips offer convenience of use and acceptable purity. A review of historical data regarding negative controls in BET applications is valuable evidence that a brand of pipette tips is non-interfering.

Another exception to problematic plastics is sterile, disposable **polystyrene tubes**, which are universally accepted as a suitable, inert container for preparation of endotoxin standards. The stability of endotoxin standards in polystyrene tubes is equivalent to borosilicate tubes, in our hands. Container-related loss of CSE potency was initially attributed to adsorption by polypropylene. Later studies by others, including our lab, found that the loss was actually caused by unknown powerful inhibitors that were extracted from the polypropylene containers<sup>1</sup>. (See Lab Pointers for further information on plastic containers for CSE).

The choice of non-glass containers for collection of water samples requires validation to assure the absence of inhibitors. The ideal collection vessel would be non-breakable, heat-stable and free of BET-interfering extractable agents. Part of validation of a water system is the identification and validation of specific non-interfering containers for collection and storage of water samples.

A common practice in the BET lab is to use a sterile **syringe** to dispense LAL reagent, such as Eppendorf® brand of 5-mL Combitip®. It is our experience that these devices are endotoxin-free, as indicated by consistently obtaining non-reactive negative controls in gel-clot and kinetic BET studies. We have been alerted to LAL-contamination problems with similar devices made by other suppliers. It is also our experience that the use of a 0.5-mL Combitip® for inoculating  $10 \mu\text{L}$  endotoxin spikes is the most accurate, efficient and robust method for preparing hot-spike positive controls in gel and kinetic BET methods.

## Microplates

Polystyrene microplates may be the item of most interest for quality control because more than one-half of all LAL tests are conducted in a microplate. A clean environment is needed for microplate fabrication to avoid dust and dirt that convey endotoxin and LRG. Some brands of microplates are troubled by the occurrence of “hot wells” where there seems to be random occurrence of over-reactive wells.

A suitable microplate for LAL work will not yield non-specific gels or hyper-reactivity in samples or standards during incubation. Therefore, a screening procedure for a microplate supplier should assure that microplate reactivity is less than lambda, the lowest concentration in a kinetic BET series. The screening procedure used by Charles River Laboratories to release sterile microplates and certify non-reactivity is the following:

1. Randomly add lambda to 4 wells;
2. Add LRW to the remaining wells; and finally,
3. Add LAL reagent to all wells.

A microplate meets the acceptance criteria if the onset times for 99% of the wells containing LRW fail to react at a time less than the wells containing lambda concentrations.

## Summary

Certain plastic accessories cause contamination of LAL reagent and release inhibitors that modify the dispersion of CSE in working standards. Polypropylene pipette tips, polystyrene tubes and polystyrene microplates are available that have non-interfering properties. Glass pipettes are the best option for rehydration of LAL reagent.

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## Meet a Few of Our European Field Technical Specialists

*Our Field Technical Specialists are highly trained individuals with a wealth of knowledge and experience with LAL testing. They work with customers daily to solve interference issues, train on new test methods and answer questions.*



Monica Vizzi is based in Italy and has been involved in LAL testing since 1999. She has a degree in biology and worked at Biologik-B in endotoxin products technical sales prior to coming to Charles River. She works very closely with customers to solve technical problems and enjoys organizing LAL congresses, workshops and training courses. She has particular expertise with software and instrument installations and qualifications as well as product validations.



Cédric Bianchi-Amoretti provides technical support in France since coming to Charles River from Amilabo where he was a technical specialist for biological indicators used by pharmaceutical companies for injectable products and medical device companies to monitor steam, ethylene oxide gas, dry heat, hydrogen peroxide, and radiation sterilization processes. He enjoys problem solving and having technical discussions with his customers.



Sascha Bär started as an Application Specialist with Charles River in Germany providing technical support for the kinetic readers and training on the software. In his current role, he provides hands-on technical support for all LAL products to his German customers. He worked as a molecular biologist and technical applications specialist prior to coming to Charles River.

## WHAT'S NEW



### Charles River Laboratories Biopharmaceutical Services (BPS)

Many of our LAL customers are not aware of the range of Biopharmaceutical Services we offer to support clients throughout the development cycle of their product, from biosafety and characterization through preclinical safety and Phase I clinicals to lot release testing.

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