Over the past 30 years, the terms “endotoxin testing” and “FDA licensing” have become so tightly associated that they are now viewed by the majority of the pharmaceutical and medical device industry as inseparable. One popular misconception is that because endotoxin testing is a final release test for human parenteral drugs, biologics and implantable medical devices, the FDA regulates the test by licensing its manufacturers.

But, it’s just not true. Final release testing and FDA licensing do not go hand-in-hand. In fact, endotoxin testing is the only final release test, universally associated with the manufacturing of injectable drugs and medical devices, that is FDA licensed. All other final release tests, i.e. sterility tests, are not.

In fact, prior to 1973 endotoxin tests, or more correctly pyrogen tests, weren’t FDA licensed either. But in the late 1960s and early 70s, the use of the LAL test to detect Gram-negative bacterial endotoxin was gaining momentum, and pharmaceutical companies were petitioning FDA to allow its use. As a result, in a notice in the Federal Register on January 12, 1973 (38 FR 1404), FDA announced that ...

“Limulus Amebocyte Lysate (LAL) derived from the circulating blood cells (amebocytes) of the horseshoe crab, is a biological product, and as such, it is subject to licensing requirements as provided in section 351 of the Public Health Service Act (42 U.S.C. 262).”

Note that it was not the fact that LAL was being considered as a means to detect bacterial endotoxin in the context of an end-product release test that initially caused it to be subject to FDA licensing. It was its source. This led the way for FDA to begin licensing LAL manufacturers and all the different LAL detection methods they would develop.

Adding to the linkage even further was the fact that up until January 2000, the United States Pharmacopeia’s Chapter <85> Bacterial Endotoxin Test only contained information on one of the licensed LAL methods, gel-clot, while LAL manufacturers were quickly moving towards quantitation. The various industries that use LAL clamored for additional guidance. The end result was the publishing of the Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-

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Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices. The guideline contained 1) formulas to calculate endotoxin limits for pharmaceuticals and medical devices, 2) validation requirements for the majority of LAL methods as well as 3) routine test requirements. In addition, the guideline stated:

“For the purpose of this guideline, the terms “lysate” or “lysate reagent” refer only to Limulus Amebocyte Lysate licensed by the Center for Biologic Evaluation and Research.”

Endotoxin testing and FDA licensing have been strongly linked ever since. The general practice has been that all reagents obtained from the blood of the horseshoe crab are submitted to CBER for licensing prior to their manufacture and/or commercial distribution in the United States.

But it is of value to note that while the need for pharmaceutical and medical device companies to test their products for bacterial endotoxin probably will not go away, the use of LAL might; and in fact has! In continuing our role as the technology leader in the endotoxin detection market, over the past several years we have developed a bacterial endotoxin test, PyroGene®, which detects endotoxin by using only a recombinant form of the endotoxin sensitive protein, Factor C. In LAL, Factor C, present in the blood of the horseshoe crab, is the first component that combines with endotoxin and initiates the reaction. In the PyroGene® assay, recombinant Factor C does the same thing, only it doesn’t come from horseshoe crab blood.

As we completed all our internal requirements prior to marketing a new product, we began to question how exactly FDA would choose to regulate this product. Since blood is not the source of recombinant Factor C, will it still be classified as a biologic and will CBER still require a license submission? Or, would CDRH, the Center for Devices and Radiological Health, be a more appropriate Center since it more often oversees in vitro tests?

To help clarify the situation in our own minds, and help us to direct our submission to the appropriate Center, we submitted a request for designation (RFD) to the FDA in December 2002. An RFD essentially requests the Agency to assign a Center with primary jurisdiction for reviewing the appropriate premarket documentation. We received a response from the Agency in a letter dated February 24, 2003. We were quite surprised and unprepared. The letter from the FDA states in part . . .

"According to the RFD, PYROGENE works in a manner similar to the Limulus Amebocyte Lysate (LAL) endotoxin test, except that the LAL test relies on Factor C derived from horseshoe crabs, whereas PYROGENE uses a recombinant Factor C. All other components of PYROGENE are synthetically produced. THE RFD states that the endotoxin detection limit and linear assay range for PYROGENE are comparable to currently marketed LAL tests. Currently marketed LAL tests are reviewed and regulated by FDA’s Center for Biologic’s Evaluation and Research (CBER). The RFD requests that PYROGENE be assigned to the Center for Devices and Radiological Health (CDRH) since PYROGENE does not make use of any live animals, and CDRH has developed relevant expertise through its review of other in vitro diagnostic products.

“CBER has regulated LAL tests for many years because of the possibility of variation inherent in animal-derived products, and because these products are intended to test blood and/or blood products for endotoxin contamination. CDRH regulates in vitro diagnostics intended for use in clinical diagnosis and patient management.

“We have carefully considered the information in the RFD and discussed the issues raised with staff in both Centers. Because PYROGENE is used to test for endotoxin contamination in other products and not man or animals, is not intended to qualify blood or blood products, and is not intended for use in patient management, we conclude that it does not require premarket approval. Accordingly, no premarket submission to either CBER or CDRH will be required. Please note that this conclusion is based on our understanding of the characteristics of PYROGENE listed above in this paragraph.”
The link between endotoxin testing and FDA licensing has been severed.

Based on the content of this letter and having completed our internal Quality Assurance requirements, we began marketing PyroGene™. The biggest question we've had from potential users is “Since PyroGene is not licensed, can we still use it for final product release in place of LAL?” The answer is an emphatic YES.

The fact that PyroGene is not FDA licensed does not in any way preclude its use as an end-product endotoxin test. PyroGene can be treated like any other QC test you use in the lab. Prior to its acceptance and routine use, its performance needs to be validated, just like any other QC test you use in the lab. USP <1225> Validation of Compendial Methods gives guidance on how methods designed to test for compendial attributes should be validated. We have prepared a validation document for your use which discusses and documents each of the validation requirements except one. And that one is product compatibility. Now, demonstrating that endotoxin can be successfully detected in your product is the only obstacle, to overcome.

After overcoming the paradigm that endotoxin testing and FDA licensing are separable, the path to the future of endotoxin testing is clear.

What’s New . . .

The PyroSense System

Cambrex Bio Science Walkersville previewed a new on-line endotoxin detection system at the fall PDA show in Atlanta. This new product allows in-process monitoring of fever-causing Gram-negative bacterial endotoxin in “water for injection” (WFI) systems used for the production of injectable drugs, medical devices, and other therapeutic products. This advanced and innovative detection system addresses FDA’s Process Analytical Technology initiative aimed at improving the current level of quality assurance for products manufactured by the pharmaceutical and medical device industries.

PyroSense™ automatically monitors endotoxin levels in WFI and high purity water systems. This robust and reliable system is designed to test round the clock – 7 days per week. The 21 CFR Part 11 compliant host software stores raw data and test results, provides immediate feedback on water quality across a company network, and provides an efficient tool for trending endotoxin levels.

This new technology moves endotoxin testing from the lab bench to the water loop and replaces the manual collection and testing of samples with an automated process. PyroSense provides consistent testing techniques using robotics and a snap-in reagent cartridge for replenishing supplies. It allows more frequent WFI testing at key points in the process without increasing labor. And it reduces risk to finished products by providing results when you need them where you need them.

PyroSense is expected to be ready for sale by fall of 2004.

For more information please contact Janet Geyer, Global Marketing Manager for Endotoxin Detection, Cambrex Bio Science Walkersville, Inc. (301-898-7025 x 2265, janet.geyer@cambrex.com).
Do you need an expert in endotoxin detection?

By Carol Roemer

The LAL Testing Service at Cambrex Bio Science Walkersville offers a wide range of testing services from basic endotoxin determination in a sample to extensive product validations.

The LAL Testing Service is drug cGMP compliant and provides expertise in gel-clot, kinetic chromogenic and kinetic turbidimetric assays. All testing is performed in accordance with the FDA Guideline and USP <85>. The Testing Service offers Preliminary Screening for a new product, which determines the concentration of the sample that is compatible with the assay and is performed prior to the product validation testing. The Product Validation Testing is inhibition/enhancement testing and is performed to show that the product can be tested on an assay at a selected concentration or dilution. Once a product is screened and validated, the testing and associated documentation can be forwarded to your lab for future Routine Testing, or you may choose to submit your product to the Testing Service for Routine Testing.

In the near future the Testing Service will begin to offer testing using Cambrex’s new PyroGene® Assay. In addition, since the PyroGene Assay contains no horseshoe crab blood, you may see us under a new name – The Cambrex Endotoxin Testing Service.

For those customers requiring immediate results, the Testing Service offers a STAT Test which provides results in 24 hours.

A more basic approach to testing is also offered, which is endotoxin determination of a sample, such as a research sample, bulk ingredient, in-process or final product sample. These samples do not undergo preliminary screening or complete product validation but are tested for inhibition and enhancement.

The Testing Service also offers testing using β-G Blocker to test for the presence of glucans in conjunction with the other services. This testing makes use of our proprietary β-G-Blocker which effectively and specifically blocks any interference from β-(1,3)-glucans that may be present in some samples.

For those customers that have submitted samples to the Testing Service, we appreciate your business. For those who have not, why not give us a try?

At Cambrex, we are focused on your endotoxin testing requirements – now and in the future.
Q: Which endotoxin detection method should I use?

A: There are a few different things you should consider before deciding which endotoxin detection method to order.

First of all, how many samples will you be testing, and how frequently? If you are going to be testing one or two samples, one time only, then you may want to consider using our Endotoxin Testing Service.

If you plan to test on a regular basis, then you first need to evaluate the equipment that you currently have. (See below: “What equipment do I need to run my assay?”)

If you have multiple types of equipment available for your use, consider the type of results you wish to obtain. If you must have a quantitative result, then realize that gel clot methods will not suit your needs. Gel clot tests are capable of giving only a “less than” or “more than” result. By running multiple dilutions of a sample, you may be able to narrow your sample’s EU/ml result down to a range, but gel clot methods are, at best, semi-quantitative.

Assay sensitivity is another important consideration. Our kinetic chromogenic LAL assay is our most sensitive: Kinetic-QCL® can read down to 0.005 EU/ml. PYROGENT-5000® and PyroGene can both read to 0.01 EU/ml, and QCL-1000® can read to 0.1 EU/ml. PYROGENT™ gel clot can be ordered in various sensitivities: 0.03, 0.06, 0.125, and 0.25 EU/ml. Many customers have the option to use either QCL-1000 or PYROGENT. If this is the case, decide if sensitivity or a quantitative result is more important to you.

If you will be testing many samples frequent basis, then you may want to consider purchasing equipment and software that you do not already have.

Our kinetic assays and PyroGene™ require less hands-on time than PYROGENT gel clot or QCL-1000, with the benefit of increased sensitivity.

We sell both kinetic and fluorescent microplate readers, as well as our WinKQCL software specifically written for endotoxin-detection tests.

Q: What equipment do I need to run my assay?

A: Each endotoxin detection assay requires the use of specific equipment. If you are running one of our PYROGENT Gel-Clot assays you’ll need to have a non-circulating water bath or a dry heat block able to heat at 37° C. If you are running our QCL-1000 assay you’ll need a dry heat block able to heat at 37° C as well as a spectrophotometer or an ELISA reader that can read at 405-410 nm. If you are running one of our kinetic assays, Kinetic-QCL or PYROGENT 5000, you’ll need an incubating kinetic plate reader and software. This plate reader should have a shaking function, be able to incubate at 37° C for the duration of the assay, and take kinetic readings at 405nm (for Kinetic-QCL) or 340nm (for PYROGENT-5000). If you are running our PyroGene assay you’ll need an incubating fluorescence microplate reader with a 380/440nm filter set. All of our endotoxin detection methods require the use of a vortex mixer and standard laboratory pipettors.

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The Q&A Corner

By Christine Hanson & Erin Passwater
Q: Can I get an MSDS for my endotoxin detection kit and/or my accessory products?

A: All of our endotoxin detection products have been evaluated and deemed non-hazardous in accordance with 29 CFR 1910.1200, the Hazard Communication Standard, based on the percentage quantities of its constituents. Therefore, none of our endotoxin detection products require an MSDS.

Q: Why should I use glassware instead of plastic?

A: Endotoxin adheres to plastic surfaces more strongly than to glass surfaces. Consequently, we recommend that you use only borosilicate glass dilution tubes when preparing your Control Standard Endotoxin (CSE) dilutions.

In some cases, certain plastics may prove to be acceptable sample preferred sample container material, polystyrene may be a good second choice, and polypropylene a third option. Some customers also report good results using PET. If you plan to store your sample for any period of time prior to testing, you should validate sample storage in the type of container you plan to use and verify that any endotoxin present in the sample will be recoverable after storage.

Q: Why is it important to vortex my CSE dilutions?

A: Endotoxin will adhere to glass surfaces, but this can be counteracted with proper vortexing to ensure that the solution you aliquot into your reaction tubes or microplate has the proper EU/ml concentration. As our package inserts state, the CSE vial should be vigorously vortexed for 15 minutes prior to making dilutions. This should be repeated each time you use the vial, not just after reconstitution.

Each CSE dilution should be vortexed for at least one-minute. It is also a good idea to vortex the CSE dilutions again, for a few seconds, immediately before use.

Q: Why should I use “matched” reagents?

A: You need to use “matched” reagents in order to comply with FDA requirements for endotoxin testing.

Each LAL lot is tested for functionality using the current United States Reference Standard. Cambrex then “matches” this LAL lot to a lot of our Control Standard Endotoxin (CSE) by testing in parallel with the referenced standard endotoxin (RSE). This RSE/CSE correlation assay determines the potency of that lot of CSE when used with that lot of LAL. Procedures for the RSE/CSE correlation assay are taken from the FDA’s Guideline on the Validation of the Limulus Amebocyte Lysate Test as an End Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices.” The FDA has stated that the use of a certificate of quality from the LAL manufacturer exempts a firm from having to perform the RSE/CSE comparison on their own. Cambrex makes your endotoxin testing simpler by offering kits containing “matched” reagents for all of our assay types.

Q: How should I handle the LAL reagent?

A: LAL reagent should be handled gently. After reconstitution, the contents of the vial will easily go into solution by either gently inverting or swirling the vial. Vortexing or vigorously shaking the reconstituted LAL reagent will cause it to foam. Reconstitute a few minutes prior to use, and let the vial sit on the bench top undisturbed until use. Never vortex your reaction vessel after adding the LAL.
Q. How should I prepare my sample for testing?

A: There are a few ways in which your sample can be prepared before testing it. Most samples only need to be diluted before they are tested with one of our endotoxin detection kits. In order to determine how far out you should dilute your sample, you should calculate the MVD (Maximum Valid Dilution) for the sample. The MVD of a sample is its endotoxin limit (in EU/ml) divided by lambda, the lowest standard of the standard curve you are using (for gel-clot, lambda is the labeled sensitivity of the lysate). You should not exceed the MVD for your sample, and to ensure a margin of safety, we recommend that you do not dilute past ½ MVD whenever possible.

We recommend heat inactivation of the sample if you suspect that proteases are interfering and causing a false positive result. This can be achieved by heating a dilution of the sample at 70 degrees C for 5-15 minutes. Further dilutions can be made from the inactivated sample.

We recommend using our Beta Glucan Blocker if you suspect contamination by Beta Glucans. This contaminant can come from yeast and cellulosic materials. A common dilution/response pattern is seen in the LAL test with samples that been contaminated by Beta Glucans. This includes a negative response with concentrated samples, a positive response with increasing dilution and an eventual negative response at the highest dilution. Additionally, with kinetic methods, a synergistic response (enhancement) is frequently seen in Beta Glucan contaminated samples.

We recommend using Pyrosperse with samples for which endotoxin binding is a suspected source of inhibition. Pyrosperse can be used with such samples as lipid emulsions, electrolyte solutions, normal serum albumin and plasma protein fractions.

Q. What pH should my sample be? What should I adjust it with?

A: Our endotoxin detection assays are optimized to work with samples with a pH range of 6-8. If the pH of your sample falls outside of this range you can adjust it with a buffer, or HCl or NaOH. Make sure the buffer you are using is endotoxin free by running it as a sample in your assay. A good choice is Cambrex's 50 mM Tris Buffer which is tested to contain less than 0.005 EU/ml.

Q. The temperature of my refrigerator/freezer has been out of range, are my reagents ok to use?

A: The normal storage temperature for LAL products is 2-8° C. Lyophilized components are very stable, but you should discard any unused reconstituted reagents. We recommend performing an initial qualification assay to confirm that the reagents still meet the performance characteristics required by the FDA.
We have recently released a new website dedicated to Endotoxin Detection. This website is focused on the needs of the endotoxin detection testing community worldwide. Highlights include:

- Links to seminars and workshops
- Product instructions
- Past issues of this newsletter
- A new “Ask Dr. Ron” section for you to post your Endotoxin Detection questions.
- Electronic purchasing

Visit us at www.cambrex.com/lal