ENDOSAFE TIMES

Endotoxin and Microbial Detection Newsletter

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PERSPECTIVE *James F. Cooper, PharmD, Consultant and Founder of Endosafe, Inc.*

Without the patient, skilled leadership of Terry Munson, there would be no LAL Test Guideline. I recently asked Terry to share his remarkable story with the LAL technology community. He sought out the best minds outside and inside the FDA to help him craft this milestone document. In the process, Terry and Christine Twohy created the first database for endotoxin testing of a wide variety of parenterals. He tirelessly pushed the document through the bureaucracy, often redrafting to overcome obstacles, and finally, obtaining agreement among all agencies. Few government servants leave such a lasting legacy.

The heart of the Guideline was the concept of an endotoxin limit, a safe amount of endotoxin for each drug, which provided a scientifically sound way to convert from rabbit to LAL testing on a dose-per-weight basis. Terry identifies two issues for which there was no scientific basis, standard curve linearity and the tolerance limit for intrathecal drugs. Evidence suggests that both should be more restrictive. Ironically, this story appears just as the FDA proposed a new BET guidance that would retire the 1987 Guideline. (Federal Register / Vol.75, July 19, 2010, p41871) Wisely, it harmonizes with the compendial Bacterial Endotoxins Test to avoid redundant testing. Implications of the draft will be discussed in the next Newsletter.

Terry now provides worldwide consultation to the parenteral drug industry as Senior Compliance Specialist for PAREXEL Consulting. He served on the US Pharmacopeia Microbiology Committee and numerous harmonization and ISO committees. Terry received his B.S. in Microbiology from Colorado State University before his 24 years of service with the FDA.

The LAL Guideline – The Rest of the Story

By Terry E. Munson, Technical Vice President, PAREXEL Consulting

In the mid 70s the FDA's Bureau of Biologics began the process of licensing the production of LAL reagents in anticipation of endotoxin testing as a replacement for the Rabbit Pyrogen Test (USP <151>) and interest in a diagnostic test for endotoxemia. Also, the Bureau of Medical Devices allowed release testing with an extract limit of 0.1 ng/mL; they also adopted an *E. coli* endotoxin standard for positive controls.[1] The pharmaceutical industry was very reluctant to change to the new LAL test because there were too many unknowns concerning what FDA would accept as adequate method validation and product limits.

Recognizing the advantages of sensitivity, simplicity and cost effectiveness of the new test, FDA decided to write an Agency-wide guidance document to promote the LAL test. They assembled a group of 22 people, most of which had never run the test and relied solely on the available scientific literature. The draft Guideline was issued January 18, 1980. The Guideline only covered the gel-clot method and presented a concentration-based endotoxin limit of 2.5 EU/mL (0.5ng/mL). This limit was patterned after the medical device limits.

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The industry response was unanimous that the Guideline was unacceptable and would actually discourage the use of the LAL test.

This is the point when I took over the leadership of the FDA LAL Task Force. A reduction in the number of members to four was required to make the process more controllable. I realized that except for the Centers for Medical Devices and Biologics there was very little expertise within FDA concerning the testing of pharmaceutical products by the LAL test. Unofficially I consulted with Dr. James F. Cooper (at this time associated with the Medical University of South Carolina), Dr. Frederick Pearson (Travenol), Marlys Weary (Travenol) and Dr. Ronald Berzofsky (at that time Biowhittaker). It is only through the collaborative effort by these individuals that the 1983 draft LAL Guideline was more acceptable to the pharmaceutical industry.

The drug industry's strongest objection was that the concentration-based endotoxin limit of 2.5 EU/mL wouldn't allow the majority of small-volume parenterals to be diluted sufficiently to meet such a stringent limit. We undertook a comprehensive study of a broad spectrum of injectables in order to learn about the extent of interfering factors in drug products. Christine Twohy set up LAL-test capability at the Minneapolis Center for Microbiological Investigations using gel-clot methods. From January 1980 to October 1983, FDA examined 2526 samples that came from 333 different drug products.[2] We determined that 236 of the products required some degree of dilution to overcome product-related inhibition. A two-lambda concentration of CSE was applied to find the compatible test dilution for validating the LAL test. A non-inhibitory dilution was not found for 14 products. About 3% of samples contained detectable endotoxin. The most common issue was pH; in this study pH was adjusted with dilute NaOH or HCl if a 1:1 mixture of LAL and the undiluted product was outside of a range of 6.0 to 7.5. Of course, the most common technique currently used to address pH interference is to use dilution, first, to minimize the problems associated with using acid or base adjustment.

> It could be said that the LAL Guideline was the first case showing what can be accomplished when FDA and industry experts collaborate on guidance documents.

The FDA learned a great deal about the nature of inhibition mechanisms and the extent of endotoxin contamination in parenterals. We found endotoxin in 77 samples associated with 24 different drug products, 15 of which exceeded our proposed FDA limit (K/M). Only one of the 15 was capable of inducing a fever response in rabbits.[2] In the process of the study, we developed a scheme of ten and two-fold dilutions to characterize the extent of dilution and provide data for validating a non-inhibitory test concentration.

With the advice of the collaborators listed above, and the experience gained in the aforementioned drug survey, the limit was changed in the 1983 draft to a more realistic dose-based system. More information was given on how to validate and test finished products. A formula was developed to determine the pass/fail dilution, better known as the Maximum Valid Dilution, based on the lysate sensitivity, drug dose/Kg and the Pyrogenic $Dose_{50}$ of 5 Endotoxin Units (EU)/Kg of body weight. As a bit of trivia, since every mathematical formula should have a Greek letter in it, the Greek letter L or λ was chosen for the **L**ysate sensitivity. The device section of the Guideline underwent very little change. Only the human drug, veterinary drug and biologics sections had major revisions.

Based on the comments received on the 1983 Guideline, further refinements were made to the Guideline. Again, these refinements were an unofficial collaborative effort between the same outside experts and me. During the next five years the Guideline was finalized and approved by all four FDA Centers covered by the guideline. This process took almost three years to finalize. It took another two years to get it approved by the FDA legal counsel. Obviously, the approval of the Guideline was not a high priority for any of these groups. The Guideline finally obtained all the approval signatures and was published in the Federal Register on February 19, 1988. One of the additional changes made to the limit calculation was the recognition that the body will clear endotoxin from the blood. So the limit calculation was revised to require that the dose used be the dose given per Kg of body weight over a one hour period.

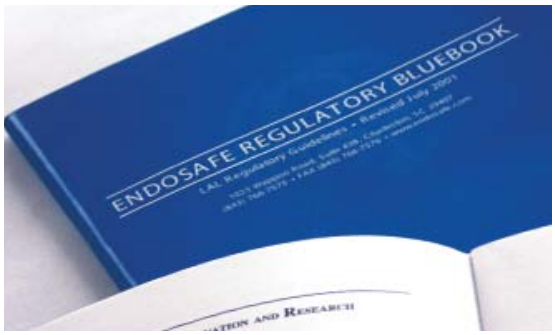
There was considerable scientific data to support the intravenous tolerance limit of 5 EU/kg. The work of Greisman[3] indicated a great similarity of man and rabbit to threshold pyrogenic levels of purified endotoxin. Further, several large studies in rabbits indicated that it was unlikely for a dose of 5 EU/kg or less to induce pyrogenic response. However, there was no corresponding data to support the intrathecal endotoxin limit of 0.2 EU/kg. Although there was data to suggest that the intraspinal route was many orders of magnitude more sensitive to pyrogens than the intravenous route,[4] a factor of 25 times more sensitive was selected because of concern that the limit would be too stringent to avoid LAL-test inhibition by dilution. The emergence of more sensitive LAL methods should prompt a new look at endotoxin limits for intrathecal drugs and compounded intraspinal infusions.

To further promote the use of the Guideline, I compiled a list of the parenteral products at that time and calculated the dose/Kg/hour and the endotoxin limit. The original list was based on rabbit pyrogen test data; however, these limits were revised to reflect human dose levels.[5] The information was obtained from the USP Dispensing Information publication and Facts and Comparison. I periodically updated the list until I left FDA in 1994. The updated lists were distributed by the LAL reagent vendors and the LAL Users Group. This role is now appropriately handled by the USP by specifications in the respective drug monographs.

LAL technology expanded dramatically during the formative years of its application and prompted revision of the LAL test Guideline soon after its release in December 1987. The glucans issue was resolved, from FDA's standpoint, by issuing a letter to the public, May 11, 1992. A non-specific activator (false positive) of the LAL reaction, LAL-RM (reactive material), was identified as a ß-D-glucan, which was found in hollow-fiber cellulose dialysis membranes and certain cellulose-based filters.[6] The letter indicated that glucans were considered more of a theoretical than an actual problem and that potential adulteration would be considered on a case-by-case basis, thus avoiding a crisis in the device industry.

The emergence of kinetic chromogenic LAL methods and incubating microplate readers presented a greater challenge. The original LAL Guideline was written with endpoint LAL methods and a kinetic tube reader in mind. Kinetic chromogenic methods utilizing multi-log ranges required new guidance for preparation of standard curves and positive controls for purposes of validity. On July 15, 1991 an unofficial Interim Guidance was issued to clarify the requirements for the Kinetic LAL Techniques. This document never went through the official rule making process and was never incorporated into the Guideline. After I left FDA there was no one to take over for me and keep the Guidance up to date.

Generally speaking, the basic principles of the Interim Guidance have served the parenteral drug industry well. The basics were incorporated into the harmonized BET, circa 2001. Regarding the linearity requirement for kinetic standard curves, I wish to clarify that the value of -0.98 was set in the absence of sufficient data and was my best guess as to its suitability. I would encourage tighter in-house linearity specifications to assure more robust assays and less invalid results attributable to standard curve related enhancement. The Pass-Fail Cutoff described in the Interim Guidance was suggested to help determine an appropriate spike concentration for products that contain native endotoxin. For the vast majority of parenteral products that never contain endotoxin, the Pass-Fail Cutoff is an unnecessary and nuisance calculation. It is advisable to exempt such products from this suggestion in kinetic LAL methods.



It could be said that the LAL Guideline was the first case showing what can be accomplished when FDA and industry experts collaborate on guidance documents. The next FDA document that went through this process, only officially, was the 2004 Aseptic Processing Guideline.

During the development of the final Guideline the USP developed a General Chapter for LAL testing, USP <85> Bacterial Endotoxin Test. This chapter was harmonized between the United States Pharmacopeia, European Pharmacopeia and the Japan Pharmacopeia. It includes all of the current techniques and gives the procedures to validate and run the different techniques. At this point in time I do not think there is a need to revise the Guideline. Since the FDA Guideline is out of date with the current LAL technology, and since the major world pharmacopeias will keep the harmonized BET Chapter up to date, it is my opinion that the FDA Guideline should be withdrawn.

Now you know the rest of the story.

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LABORATORY NOTEBOOK

A Study of Interlaboratory Variation in the Bacterial Endotoxins Test

Masakazu Tsuchiya, Ph. D., Gunther Mieke, Guy Mbaya, Alan Hoffmeister - Charles River

Overview

Consistently reliable test results don't happen by accident. Leading laboratories utilize regular assessment to ensure they maintain the utmost confidence in every analysis. An element of this assessment is participation in Proficiency Testing Programs (PTP). Proficiency testing is a powerful and important quality assurance tool that enables laboratories to compare their analytical measurements against both themselves and peer laboratories over time. There are numerous benefits to laboratories participating in PTP, such as monitoring of measurement trends, detection of bias, validation of methods, demonstration of competence to external auditors, proficiency feedback to analysts, method improvement and provision of data to identify best practices and problem measurements.

In 1993, the first LAL Proficiency Testing Program was instigated in France by the Amilabo group. Initially a domestic program, it soon gained acceptance across Europe, allowing participants the opportunity to assess their analysis against European counterparts and their methods against the day's regulatory requirements. From its inception, the LAL PTP has been open to all, irrespective of method or brand of reagent used. With participating laboratories continuing to increase, Charles River became the custodian of the PTP with the acquisition of the French originators and has continued to develop the program into a worldwide tool. With over 320 laboratories from countries all around the globe participating, the LAL PTP remains the foremost international quality assurance tool available to the LAL community.

Introduction

The Limulus Amebocyte Lysate (LAL) test is recognized as a quick and sensitive method for detecting endotoxin. Although LAL test methods are established for the Bacterial Endotoxins Test (BET) in Pharmacopoeias, discrepancies in the endotoxin assay between laboratories are sometimes encountered. There are few articles to show the practical variability of the BET. This article will try to analyze the current situation of the BET and to pinpoint the errors in the practical LAL test. For this purpose, the results of the Proficiency Testing Program (PTP) from 2008 were analyzed.

What is PTP?

PTP is a program for LAL users to provide a confidential audit of their LAL test proficiency. Control samples are sent quarterly to the participants, and the measured values are reported back to Charles River. Control values are determined by Charles River using US Reference Standard Endotoxin and the gel-clot method, and the potency is confirmed with all other methods. PTP is open to any LAL user wanting to verify working procedures through an external source. The analyst's methodology, results and report are examined for accuracy in accordance with European and US Pharmacopoeia regulations, and a confidential report with detailed results from the audit is sent back to the laboratory.

Reported values for the control samples

There were four sessions of PTP in 2008, and a total of 1159 results were collected. Table 1 shows the basic statistics of the sessions. The reported values from the participants contain irregular values. In the case that some of the irregular values are too far from the actual values, the mean of the reported values could be affected. To avoid this type of error, medians of the reported value could be appropriate to determine the typical values of the PTP control assayed. Figure 1 is a plot of the ratio of reported values of the control samples for the following LAL methods: gel-clot (GEL), kinetic chromogenic (KCA), kinetic turbidimetric (KTA), endpoint chromogenic (EPC), and the Portable Test System (PTS), which uses a pre-calibrated LAL cartridge. The control values established by Charles River were set as 100%.

Since there were five methods to be compared and the values for each method seemed not to have equal variances, the Kruskal-Wallis Test was applied for the statistical analysis. The Kruskal-Wallis Test indicated significant difference between the averages of the ratio of the values for BET methods ($p < 0.0001$). The GEL seemed to be the method with the most significant difference. Although there was a significant difference among the methods, the median and mean values for each method were between 50% and 200%.

TABLE 1: BASIC STATISTICS OF 2008 PTP

Method/ Quarter	Control Value (EU/mL)	Median (EU/mL)	Geometric Mean (EU/mL)	Mean (EU/mL)	SD (EU/mL)	CV	Max (EU/mL)	Min (EU/mL)	Participant
GEL A08		0.125	0.157	0.187	0.101	64%	0.600	0.001	62
KCA A08		0.142	0.133	0.150	0.066	44%	0.413	0.009	68
KTA A08	0.197	0.140	0.151	0.279	1.130	405%	10.121	0.052	78
EPC A08		0.146	0.174	0.210	0.163	77%	0.450	0.100	4
PTS A08		0.123	0.114	0.119	0.031	26%	0.163	0.049	15
GEL B08		0.500	0.480	0.534	0.255	53%	1.500	0.125	95
KCA B08		0.433	0.449	0.479	0.181	38%	1.090	0.135	90
KTA B08	0.424	0.444	0.442	0.462	0.141	31%	0.920	0.147	80
EPC B08		0.380	0.380	0.438	0.254	58%	0.989	0.126	w10
PTS B08		0.317	0.282	0.293	0.075	26%	0.482	0.126	27
GEL C08		1.000	1.414	1.662	1.139	81%	8.000	0.480	61
KCA C08		1.314	1.282	1.362	0.480	35%	2.640	0.506	88
KTA C08	1.51	1.370	1.306	1.366	0.406	30%	2.600	0.463	70
EPC C08		0.680	0.661	0.680	0.226	33%	0.840	0.520	2
PTS C08		0.893	0.897	0.952	0.284	30%	1.640	0.175	26
GEL D08		2.000	2.356	3.030	2.892	123%	22.620	0.125	106
KCA D08		1.927	1.851	2.009	0.739	37%	4.140	0.100	120
KTA D08	2.52	2.196	2.126	2.300	0.821	36%	5.060	0.140	126
EPC D08		1.640	1.656	1.713	0.460	27%	2.463	0.957	7
PTS D08		1.600	1.625	1.795	1.168	65%	7.050	0.644	24
								TOTAL	1159

Comparison of the Pass/Fail ratio of the methods

The results were classified in 4 different standpoints:

1. **Pass/Fail**: Pass or fail results judged by the criteria of PTP.
2. **Wrong Value:** Measured values not in the range of 50% to 200% of the control value.
3. **Technical Error:** Measurement-related errors, such as unsuitable results in negative controls, positive controls, positive product controls, linearity of standard curves, or confirmation of labeled sensitivity. This includes Wrong Values. This indicates unsuitable measurement techniques for the LAL test.
4. **Procedural Error:** Unsuitable procedures for replicate number of samples, unsuitable spike concentration for positive controls, unsuitable range of standard curves, or failure of submission of the certificate of analysis for the Control Standard Endotoxin. This indicates unsuitable procedures or misunderstanding of the BET.

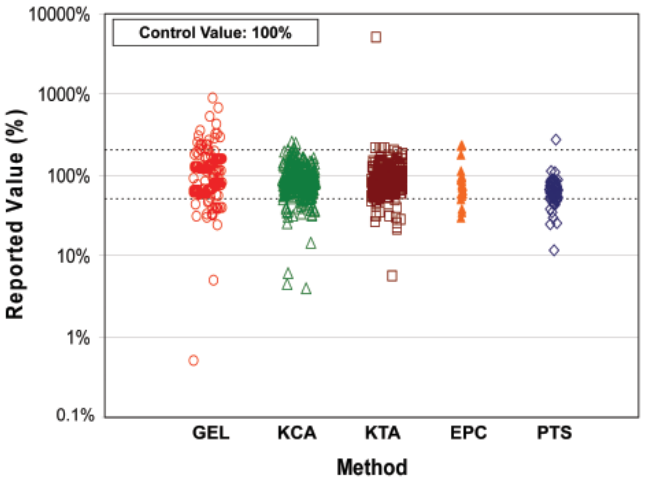

FIGURE 1: Ratio of Reported Values in 2008 PTP

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TABLE 2: SUMMARY OF THE FAILURE IN 2008 PTP

Method	Participant	Failed Total		Wrong Value		Technical Error		Procedural Error		Technical and Procedural Error	
GEL	324	108	33.3%	54	16.7%	67	20.7%	57	17.6%	16	4.9%
KCA	366	77	21.0%	33	9.0%	51	13.9%	29	7.9%	3	0.8%
KTA	354	53	15.0%	24	6.8%	47	13.3%	15	4.2%	9	2.5%
EPC	23	11	47.8%	5	21.7%	10	43.5%	6	26.1%	5	21.7%
PTS	92	14	15.2%	14	15.2%	14	15.2%	0	0.0%	0	0.0%
TOTAL	**1159**	**263**		**130**		**189**		**107**		**33**	

Table 2 shows the summary of the failure. Overall Pass/Fail results (Failed Total) showed high failure rates in the EPC and GEL. There was significant difference in the failure ratio of the methods according to the chi-square test ($p < 0.0001$), with the GEL having the highest chi-square value. There was significant difference in the Wrong Value of the methods according to the chi-square test ($p = 0.0002$).
Once again, the GEL was the main contributor to the significance, according to the chi-square value. There was significant difference in the Technical Error of the methods. In this case, the EPC was the method that most contributed to the significance. The Procedural Error of the methods also showed significant difference. The GEL was the method most responsible for this significance.

Discussion

There was significant difference in the values measured among the BET methods. However, the median and mean values of each method were in the range specified in the Pharmacopoeias (between 50% and 200%). There are several factors that can cause bias, such as the vial-to-vial variability of the endotoxin standards, quality of water used, and vial-to-vial variability of LAL reagent. Since it is sometimes hard to control them, it is reasonable to set the acceptance range between 50% and 200% for BET, which is a biological assay.

Technical and procedural errors were frequently found in the GEL and EPC. The GEL is a manual method and probably has more human error than other methods. The EPC seemed to have similar errors. The GEL is a semi-quantitative method. The resolution of the assay is usually 2-fold, and the true sensitivity is usually higher than the labeled sensitivity (Figure 2).

This may cause bias for the control value of the PTP. Considering this analysis, a method with less manual procedures is preferable for the BET.

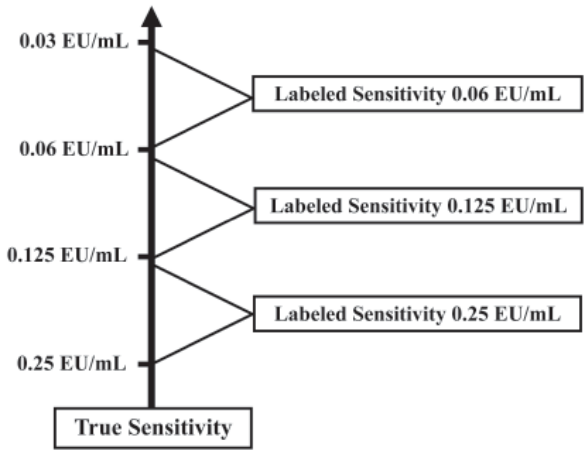


FIGURE 2: Relationship Between True Sensitivity and Labeled Sensitivity of LAL

Part of this article was presented at the PDA Annual Meeting in 2010.

LAL POINTERS

Why it Makes Good "Cents" to Use Certified Laboratory Accessories

Our Technical Service Group often receives calls from customers about assays that "didn't work". In the majority of these cases, the cause of assay failure is due to either a lack of understanding about running the LAL test (which is attributed to incomplete training) or inadequate laboratory accessories such as microplates, pipettes and water that don't meet the minimum requirements for the assay.

In these trouble-shooting discussions, we always recommend the purchase of certified accessories from reputable suppliers, preferably the vendor who supplies the LAL reagent. This recommendation, in many cases, is looked upon as a way to generate more revenue, but this is not the basis for the recommendation. These accessories are certified and labeled as endotoxin-free and free of interfering factors and are absolute requirements for a robust assay that meets the sensitivity requirements of the test.

When compared to standard laboratory accessories, these validated products are more costly and difficult to justify to Purchasing Agents. However, is it worth saving $2.50 on a microplate and risking an invalid assay because your negative controls reacted due to the contaminated plate? We have summarized the projected expense and time of a typical kinetic chromogenic assay. As Dr. James Cooper, the founder of Endosafe once said, "The most expensive test is the one that needs to be repeated", and we couldn't agree more.

Cost and Time Considerations for a Typical Kinetic Chromogenic LAL Assay

Reagents and Consumables Required for the Test	**Labor Involved in the Test** *(time to complete steps)*
CSE	Sample preparation *(30 minutes)*
Vials of Endochrome-K LAL	Preparation of standards *(30 minutes)*
LAL Reagent Grade Water	Plate preparation *(15 minutes)*
Microplate	LAL rehydration *(15 minutes)*
Pipettes	Assay run time and data analysis *(90 minutes)*
Pipette tips	

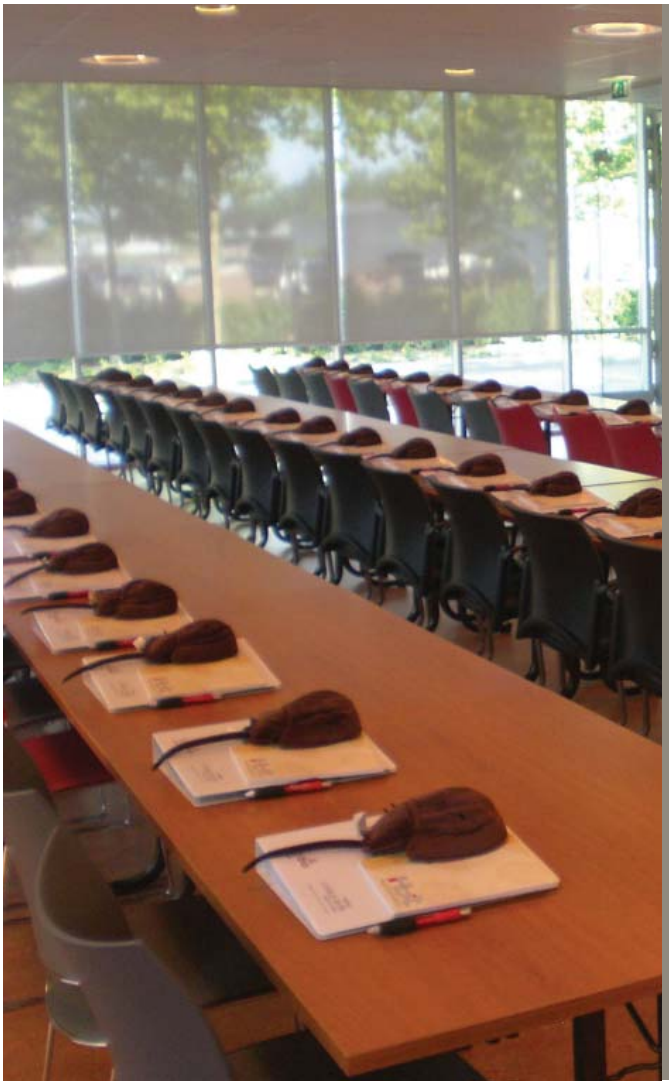
Standard total cost for the assay: $365 US, 577€ • Total time invested: 180 minutes *(3 hours)*

WHAT'S NEW

Publication Compares PTS™ Rapid Endotoxin Test Method to Gel-Clot Assay

Operators in the microbiology laboratory at Immunomedics evaluated the Endosafe®-PTS™ and the gel-clot assay for endotoxin testing of biopharmaceutical samples including raw materials and finished products. Factors in the published study included evaluation of endotoxin values, ease of use, completion time, resource optimization and sample volume required. The study concluded that the speed and equivalent results with the PTS™ allowed optimization of their endotoxin testing program by reducing sample volume, analyst manipulations, accessory materials, turnover time and risk.

Citation: Jiminez L, Rana N, Travers K, Tolomanoska V and Walker K, Evaluation of the Endosafe® Portable Test System™ for the Rapid Analysis of Biopharmaceutical Samples. PDA *Journal of Pharmaceutical Science and Technology*, May-June 2010, Vol. 64, No. 3.



UPCOMING SEMINARS & WORKSHOPS

Charles River LAL Workshops provide LAL professionals with valuable technical and regulatory information from leading experts with extensive experience in the application of LAL testing in the pharmaceutical, medical device, biotech and dialysis industries.

With the increasing demands of endotoxin testing across all industries, there is a distinct need to advance the understanding of test technology and the skill level of those responsible for upholding the stringent requirements for LAL testing in their facilities. The Charles River LAL Workshop is an intensive experience, designed to help participants meet those needs.

Don't miss this opportunity to learn from the experts. Please join us for an LAL training event in 2010:

- August 24-27 ..... *Charleston, SC*
- September 13-14 ..... *Ireland*
- September 15-17 ..... *England*
- September 21 ..... *France**
- September 21-22 ..... *Germany*
- September 23-24 ..... *Benelux*
- September 28-October 1 ..... *France**
- November 30-December 3 ..... *France**

**The France LAL Training is conducted at a special training lab in Les Oncins outside of Lyon.*

For more information, please contact us at 1.877.CRIVER.1 (1.877.274.8371) or global-endocomments@crl.com.

A WORD ABOUT CRABS

We have just completed our most successful horseshoe crab bleeding season ever. The number of horseshoe crabs we bled this season is more than three times the number bled 10 years ago. Our yield from this year's season has provided us with a 28-month supply of LAL raw material. A large part of our success can be attributed to Charles River's unique management of the horseshoe crab population in South Carolina (the location of Charles River's crab bleeding facility). In 1992, Dr. James Cooper (our company founder) and his wife Frances initiated a dialogue with the South Carolina Department of Natural Resources. This dialogue resulted in South Carolina legislation that protects the indigenous horseshoe crab population. In South Carolina, horseshoe crabs can only be used for biomedical applications (LAL production) and marine biological research and not as bait for the eel and whelk industries. As a result, horseshoe crabs are more protected in South Carolina than anywhere else in the world.

This proactive legislation and our commitment to only "hand harvest" horseshoe crabs (as opposed to trawling) has led to a tremendous increase in the horseshoe crab population in South Carolina. Each year, the touching, handling, bleeding and processing the blood of the horseshoe crab reminds us of who we are and what we do. We continue to be awed by this magnificently ancient creature from the sea. We continue to be inspired by technology that allows us to create this fantastic reagent that has profoundly improved the quality and safety of medical products. Yet this year, all of our eyes are on the Gulf of Mexico. Our immediate concern is the environmental and economic impact this tragedy has created. We are fortunate to have enough raw material for more than two years and feel prepared to weather any storm ahead.