ENDOSAFE TIMES

IN THIS ISSUE:

PERSPECTIVE

Recent FDA approval of our new Endosafe®-PTS makes the topic in this *Endosafe Times* even more relevant. The single most asked question about the PTS is: "What is the variability of the test?" Although that may seem like a very simple question it is not. All FDA-licensed LAL test methods must meet a minimum requirement of ± 2-fold dilution of a known endotoxin standard (RSE). The Endosafe®-PTS not only meets, but exceeds this minimum requirement. To understand why, it is important to understand what influences the variability of the assay and how the design of the PTS platform minimizes those influences. We hope this newsletter will address the issues and allow you to maintain a more precise and robust LAL test method.

VARIABILITY IN THE BACTERIAL ENDOTOXINS TEST OR LAL (LIMULUS AMEBOCYTE LYSATE) TEST

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INTRODUCTION

One of the most common questions asked by users is the variability in the LAL test between different analysts, different LAL manufacturers, different sensitivities of lysate, and different methods used to test products. It can be gathered from various scientific articles and books written on the subject [1, 2] that this variability comes from three main sources–reagent, product and method.

We'll take a brief look at the LAL reagent, endotoxins and the LAL test itself to address some of these variations.

LAL REAGENT

The reagent (lysate) used in the LAL test is of biological origin and is extracted by the osmotic lysis of amebocytes found in the intracellular fluid of the American horseshoe crab (*Limulus polyphemus*). It is a complex mixture of different enzymes and co-factors that form a clot through a cascade reaction when triggered by the presence of endotoxins in the solution. Since the extract is a crude mixture and not a single purified enzyme, the enzyme activity cannot be determined precisely for every lot extracted. Although every manufacturer of LAL follows similar extraction and manufacturing procedures, they each have their own formulation from which

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LAL Reagents

lysates of different sensitivities are prepared. This includes additions of buffers, detergents, etc. which contribute to sources of variability. All licensed manufacturers have to follow their BLA filings with the USFDA to release their LAL reagents.

The enzyme activity of this formulation (labeled as lysate sensitivity, λ) is determined against a Reference Standard Endotoxin (RSE) preparation supplied by the FDA. An assay is performed by preparing a 2-fold dilution series made from 1 EU/mL of RSE. Since the lysate potency is performed in 2-fold dilutions, it contributes to a source of variation; e.g., the true sensitivity of a labeled 0.125 EU/mL lysate lot may actually be 0.10 EU/mL.

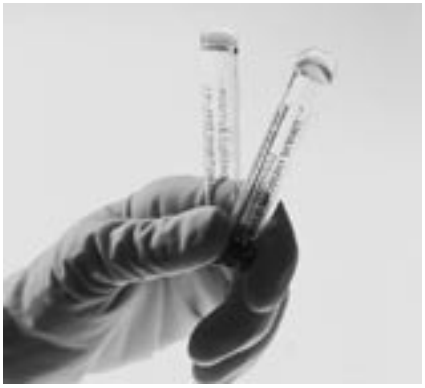
RSE, however, is not available to users because it is expensive and exhaustible. LAL manufacturers therefore supply Control Standard Endotoxin (CSE) whose potency is determined against the RSE for every lot of lysate. Each lot-specific combination of LAL and CSE must be characterized according to the method being used and a specific RSE/CSE ratio (EU/ng value) cannot be assigned until the testing is complete. This indicates that early regulations of the LAL test controlled variation in the reactivity of different endotoxins and lysate lots by a direct comparison to RSE. The RSE/CSE ratio is indicated on a Certificate of Analysis and issued with every LAL reagent and CSE Lot combination.

It is important to keep in mind that the active ingredient in the raw material is biological and that a biological assay is by default more variable than a chemical assay. Because the LAL assay is much more specific than the rabbit pyrogen assay, analysts, and reviewers tend to forget that it is a biological assay. To further point out the differences between a biological and a chemical assay, one could reference the differences in validation requirements of a non-compendial method for a chemical versus a biological assay.

ENDOTOXINS

Endotoxin used in the preparation of CSE is obtained from purified E.coli strains which have been adequately characterized. CSE is a highly purified lipopolysaccharide (LPS) preparation that is reasonably free of detectable contaminants, particularly protein. It also contains stabilizing fillers like human serum albumin, PEG, and starch, so that it performs reliably and reproducibly when used in control and standard curves. This is important in order to ensure that all other parameters of the test are within acceptable limits.

Environmental or natural endotoxins, on the other hand, are not purified and are macromolecular complexes of LPS, cellular membrane proteins



Gel-Clot Test

and phospholipids. They are constantly shed by Gram-negative bacteria into the environment during normal growth periods. Depending on the environment of the natural endotoxins, they either aggregate to form 3D supramolecular (lamellar, cubic, hexagonal) structures, or can sometimes contain even less protein and phospholipid than purified LPS [3].

Toxicity and reactivity of the lipid A moiety in the LPS (which is known to be fairly constant in all Gram-negative bacteria) has been theorized to be dependent on the given bacterial species' LPS to adopt non-lamellar, conical structures which trigger the receptors on target cell membranes to release endotoxic mediators. LPS that prefer a lamellar structure where the hydrophobic lipid A moiety is inward in the bilayer, exhibit little or no biological activity [4]. Thus, the conformation and method of presentation of these endotoxin macromolecules in solution are an important factor that contributes to varied responses of natural endotoxins to the LAL reagent. And, in order to get optimal endotoxin-LAL reactions, LAL manufacturers often modify the formulation of the reagent by adding divalent cations (Mg^{++} and Ca^{++}), buffers, detergents, etc.

LAL TEST AND LAB VARIABILITY

The LAL test can be performed by 2 broad methods-qualitatively by the gel-clot test and quantitatively by the kinetic test.

Gel-clot method

The gel-clot method includes a limit test (qualitative) and an endotoxin titer assay (semi-quantitative). In both cases, an endotoxin standard series is prepared using four concentrations of CSE that bracket the lysate sensitivity (λ). It is run at least once a day with the first set of tests and repeated if there is any change in lysate lot, endotoxin lot, or test conditions during the day. Please refer to Appendix C: 1987 FDA Guidelines or the USP <85> Bacterial Endotoxins Test for detailed procedures [5].

Endotoxin-free Accessory Products

The gel-clot test is valid when the end-point of the endotoxin standard series is within a $\pm$ two-fold dilution of the labeled lysate sensitivity (indicating the inherent 2-fold error/variability in the test). A valid endotoxin standard series determines primarily that the accessories being used are within prescribed parameters and that the positive spike (2λ concentration) used for Positive Product Controls (PPC) forms a gel-clot. The test is performed in duplicate. In all gel-clot assays, the PPC must always be positive (form a clot) to be valid.

Kinetic method

The kinetic test is performed using either the Chromogenic method or the Turbidimetric method. Both methods require a spectrophotometer and software for real time data collection as the color/turbidity increases in each well. Data is processed using specific software and results are printed out in endotoxin units.

A 2- to 4-log standard curve is prepared from CSE and the product endotoxin values are calculated based on this standard curve. The assay is performed in duplicate and a valid assay must be within specified parameters. In all kinetic assays, the PPC must have a 50 to 200% recovery of the theoretical spiked value to be valid. Typically, the slope of the RSE curve is slightly different from that of the CSE or one prepared from environmental endotoxins. These different slopes can cause variability in reported endotoxin values especially from data points close to the highest and lowest standards.

Accessories and analysts

Test tubes (borosilicate or flint), disposable pipettes, micropipette tips and 96-well microplates can all cause some variability. Analytical technique is another source of variability in conducting the test. Small variations in pipetting, preparing control standards and dilutions can give different end-points in the endotoxin series of a gel-clot test (or different linear regression data of the standard curve in kinetic assays) even though all accessories, LAL reagent lots, and LAL Reagent Water used are exactly the same.

In the kinetic test, lab accessories and techniques play an enhanced role in causing variability when low CSE concentrations are used in the standard curve (the lowest standard can be as low as 0.005 EU/mL). Polystyrene plates and tips have to be from a reliable source with proper QA certification. Pipetting errors from in-plate spiking (10 μL of CSE) of positive control wells and cross-contamination can occur easily.

Table 1. Materials

	Plastic Accessories	Glass Accessories
Standard curve R-value	-0.9868	-0.9979
Standard curve back - calculated values		
5.0 EU/mL	6.22	4.59
0.5 EU/mL	0.32	0.59
0.05 EU/mL	0.06	0.05
Known endotoxin concentration reported value		
0.5	0.85	0.62
0.06	0.1	0.058

Table 2. Slope Differences

Dilution of Tap Water Sample	Reported EU/mL	Part of Curve Reaction Time Included in.
Undiluted	6.15	50 - 5 EU
1:10	4.98	5-00.5 EU
1:100	3.6	0.05-0.005 EU
1:1000	<5.0	>0.005 EU

Product Interference

Before any product can be tested, it has to be screened for interference (both inhibition and enhancement) and a non-inhibitory concentration (NIC) determined for routine testing. Lot to lot variation in the manufacturing of products (excipients , active component, containers) can lead to variability in results due to changes in interference patterns.

Endotoxins in drug products can behave differently depending on the product-lysate environment. If there is a higher availability of the lipid A moiety because of a conformational change in the structure in solution, then it results in a more effective endotoxin-lysate reaction giving a higher endotoxin value.

Table 3. Inhibitory sample

% Recovery	Reported EU/mL for a sample with 0.5 EU/mL
69%	0.39
90%	0.50
101%	0.56
104%	0.57

Method

The LAL test is subject to an inherent ± 2-fold (50% to 200%) error which allows a valid endotoxin standard series in the gel-clot test to have an end-point between 2λ and 0.5λ. Therefore, when determining the endotoxin content in a sample by the gel-clot endotoxin titer assay, repeated dilutions *within* a 2-fold dilution to obtain a more accurate endotoxin value are superfluous and unnecessary.

The LAL test also allows an acceptable spike recovery in the PPC of 50% to 200% in a kinetic assay. In some products, differences in spike recovery are clearly seen when the same sample is tested by the gel-clot vs. kinetic method. A positive PPC by gel-clot method sometimes gives only a 60% spike recovery by the kinetic method. Although acceptable, it is seen that interferences causing borderline spike recovery can affect the quantity of endotoxin calculated by the kinetic method. In such cases, Charles River's 0.03 and 0.06 EU/mL sensitivity reagents, which can be used for both gel-clot and kinetic methods, provide a good tool to determine the appropriate NIC when changing from gel-clot to kinetic methods.

In other cases, when moving from a lower sensitivity lysate (e.g., 0.125 EU/mL) to a higher sensitivity lysate (e.g., 0.03 EU/mL), changes in the formulation of the lysate can cause variation in the amount of endotoxin detected in the product.

Endosafe®-PTS Test System

In conclusion, it must be noted that although there are sources of variability, the USP and USFDA Guidelines allow the user to be within the ± 2-fold error margin. The LAL test is done keeping this in mind. It is therefore strongly recommended that the product never be released at the endotoxin limit and that a safety factor of at least 2- to 4-fold as an internal limit be maintained, thereby allowing for the ± 2-fold error when the same product is tested at another laboratory.

The answer given by Mr. Terry Munsen (ex-FDA) to Karen McCullogh when she presented this question to him was as follows [6]:

Q. "What do you do if you test your product with 3 different lysates (from 3 different manufacturers) and the endotoxin results are different (up to 8-fold)?"

A. "Use the maximum inhibition end-point of the 3 lysates for testing. If the results are truly different, take the highest (most conservative) value."

This is a good rule of thumb to bear in mind when looking at variability in results and it is applicable across a wide variety of LAL test situations. Of course, when there is no endotoxin or very low amounts of endotoxin present in the product being tested, none of these situations apply. However, they become significant when endotoxin is present in the product, especially when the amount is close to its endotoxin limit.

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[3] Brandenburg, K., et al., Conformation of Lipid A, the Endotoxic Center of Bacterial Lipopolysaccharide. J. of Endo. Res., 1996. 3(3)
[4] Appendix C : 1987 FDA " Guideline on Validation of the Limulus Amebocyte Lysate as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices" Appendix B : US Pharmacopoeia <85> Bacterial Endotoxins Test
[5] Rietschel and et al., Bacterial Endotoxins : Molecular Relationship of Structure to Activity and Function. FASEB, 1994, Feb 18
[6] Munson, Terry E., ex-CDER/FDA, Department of Health and Human Services, 1991, Feb 5 : Answers to LAL Users Forum Questions (c/o Karen McCullogh)

Table 4. Endosafe®-PTS vs. Endochrome-K

		PTS Test Reults		Endochrome-K Test Results	
Sample Description	Dilution Tested	Endotoxin Value	PPC Recovery	Endotoxin Value	PPC Recovery
3% NaCl	1:10	<0.50 EU/mL	102%	<0.50 EU/mL	104%
PET	1:10	<0.50 EU/mL	82%	<0.50 EU/mL	118%
Protein	1:10	0.86 EU/mL	158%	1.5 EU/mL	143%
Device Extract	1:1	0.23 EU/mL	81%	0.46 EU/mL	87%
HSP 70	1:1000	290 EU/mL	104%	380 EU/mL	149%
FBS	1:100	26 EU/mL	108%	47 EU/mL	184%
FBS Lactated Ringers	1:1	<0.05 EU/mL	120%	<0.05 EU/mL	101%
Dialysate	1:10	<0.50 EU/mL	137%	<0.50 EU/mL	113%

LABORATORY NOTEBOOK

Now that FDA has approved the Endosafe®-PTS, many customers will be considering a validation test plan for many of their existing products. When designing a plan, it is important to remember that the reagents used in the PTS platform are traditional FDA-licensed chromogenic LAL reagents. It is therefore reasonable to assume that the original validated test dilution will also test successfully on the PTS. Following this approach, you should validate the PTS cartridge sensitivity that will achieve the desired endotoxin limit of your product assuming the existing sample test dilution. Your product validation on the PTS platform should include testing three lots of product at a fixed dilution not greater than the MVD. Each of the three lots should give endotoxin test results below the endotoxin limit with acceptable spike recoveries between 50-200%. Table 4 demonstrates the similarity of the PTS system to our existing Endochrome-K. Although there were no specific acceptance criteria assigned to this comparative analysis, all results were within the FDA and USP criteria for spike recovery of 50-200%. In addition, results from the PTS method are very similar to those obtained from the FDA-approved Endochrome-K test results. Samples that do not contain measurable endotoxin by the approved Endochrome-K test are negative with the PTS methodology also (absence of a false positive). Samples with endotoxin levels are within the inherent variability of the LAL methods (less than 2-fold).

LAL POINTERS

A common problem that faces new kinetic LAL users is the impact of bubbles on the optical measurements in 96-well microplate readers. Kinetic measurements are based on the delta OD change of each sample well. Bubbles created in these sample wells tend to move, break, or even expand during an assay. It is absolutely necessary to develop a technique that effectively eliminates bubble formation during sample loading in order to have a robust LAL method. With this problem in mind, the latest PTS software is equipped with software that can actually inform the users of an improperly filled sample well.

WHAT'S NEW

Addendum (IV) to the Fishery Management Plan for Horseshoe Crabs Has Been Approved

This latest addendum, published in mid-June, declares that crab population levels in the Southeast region of the Atlantic Ocean are stable. Please visit www.criver.com to download a copy of "The Horseshoe Crab Technical Report".

LAL Workshop Registration Ongoing

The Endosafe® Summer Workshop on bacterial endotoxins technology, gel-clot and kinetic methods will be held on August 22-25 in Charleston, SC. To register, please call Jill at 1.800.762.7016 or download the form off the web site, www.criver.com.

New Product Now Available

Endosafe® Microtrend, the next generation of trending software for endotoxin testing is now available. For information, please visit our web site, www.criver.com, or call 1.877.CRIVER1. The product code is M804 and the validation package is TS1000.

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