# ENDOSAFETIMES

Endotoxin and Microbial Detection Newsletter

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## PERSPECTIVE

The past forty years have witnessed the development of LAL (*Limulus* amebocyte lysate) technology, beginning with the application of the new reagent to pyrogen testing. Cooper and Mills produced the first commercial LAL reagent in Chincoteague, VA in 1971[1]. In 1973, the U.S. Food and Drug Administration's (FDA) Bureau of Biologics (now called the Center for Biologics Research and Development) published its intent to regulate LAL reagent as an *in vitro* biologic. The decision to regulate was made because of the potential of the reagent as a replacement to the rabbit pyrogen test and as a human diagnostic product for endotoxemia. The endotoxemia test never materialized, but FDA oversight of LAL manufacturing contributed to the development of stable, reproducible reagents for endotoxin detection. The LAL industry emerged in 1977 with the approval of three commercial gel-clot reagents for in-process testing. The simplistic gel-clot test has evolved into more sophisticated kinetic assays using incubating readers, novel test platforms, powerful software and automation. The feature article in this issue will review the development of endotoxin test methods and describe how innovative LAL-dependent systems became standard, compendial methods.

**Reference**


1. Cooper JF. 2008 Bacterial endotoxins test, chapter 22. In: Prince R, ed. *Microbiology in Pharmaceutical Manufacturing, Vol. 2, 2nd Edition,* PDA, Bethesda, MD.


# Evolution of the Bacterial Endotoxins Test (BET)

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

Growth of LAL-test technology was initially plagued by the lack of reliable endotoxin standards, uniform gel-clot methods and agreement on an endotoxin limit. The U.S. Food and Drug Administration (FDA) and U.S. Pharmacopeia (USP) jointly produced a 30-gram batch of an *E.coli*-derived endotoxin (0113:H10:K0) that became the source of subsequent Reference Standard Endotoxins (RSE). Lot EC5 was the first large batch (30,000 vials) that had good stability and reproducibility. Introduction of EC5 in 1982 enabled LAL manufacturers to reliably determine the sensitivity of their gel-clot reagents and calibrate their control standard endotoxins (CSE). A value of 10,000 Endotoxin Units (EU) per vial was assigned to the batch1. The FDA controls a large portion of RSE and makes this available to LAL suppliers for reagent certification purposes.

## in this ISSUE



An informational chapter for use of LAL reagent was published in *USP XX* (1980) under the title of "Bacterial Endotoxins Test (BET)." The new chapter suggested general methods for gel-clot tests and preparation of endotoxin standards. About this time, the FDA, under the leadership of Terry Munson, began drafting an LAL test guideline to enable and encourage the parenteral drug and medical device industries to switch from rabbit to LAL testing. Munson mobilized scientists from industry and academia to create a scientifically sound basis for the guideline. The endotoxin limit (maximum safe amount of endotoxin) was determined from an impressive group of rabbit fever studies. The data indicated that 5 EU/kg of the RSE was approximately the threshold dose for pyrogenicity in rabbits[1,2]. There was good evidence that man and rabbit were relatively equal in response to threshold endotoxin

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levels. Later, a study of pyrogenicity of RSE in humans substantiated this tolerance limit[3]. Although not finalized until 1987, draft guidelines influenced the orderly development of standard test methods and presented ways to validate test conditions for end-product release of parenteral products.

The LAL test guideline of 1987 contained important information in appendices. There was a reference to the current BET chapter, a method for determining the RSE/CSE ratio for calibration of secondary standards of CSE and a table of endotoxin limits for parenteral drug products. This guideline had limited information about photometric methods because chromogenic and turbidimetric assays were just being introduced to the market.

**BET Supplants Pyrogen Test**

Revisions to *USP XXIII* that became effective in Supplement 8 (1993) represented the compendial switch from rabbit to LAL methods for end-product testing for most parenterals (except blood products; controlled by a Public Health Service Act). Endotoxin limits, based on human dose, were assigned to the monographs of injectable products. The official and referee LAL test was gel-clot, only. At that time, the emerging photometric methods were allowed when suitably validated as an alternate method.

The introduction of the harmonized BET in 2001 brought momentous changes to the BET chapter[1]. All photometric analyses were now official methods. The authors of the new BET were wise to avoid highly prescriptive language that might suppress innovation. For example, the BET was silent on issues such as method of regression analysis for kinetic standard curves, archived standard curves and selection of the positive control level. With the advent of the harmonized BET, the value of the FDA's LAL test guideline began to fade. Only the BET has a mechanism for continuous review and revision through the harmonization process. After all, **it is the purview of pharmacopeial bodies to establish drug standards and the responsibility of federal agencies to enforce such standards.**

> The BET chapter is powerful because it is comprehensive without being a prescriptive document.

**BET Gel-Clot Technique**

How do we determine that a laboratory's LAL method is consistent with the BET? For a gel-clot technique, the critical elements for a routine BET include a validated method for preparing a test sample, a positive product control (PPC) to check for inhibitory conditions and selection of non-interfering test materials. From the description for a gel-clot limit test, it might appear that negative and positive water controls are required on a one-to-one basis with each sample. In practice, however, sufficient positive water controls are done at appropriate intervals to demonstrate that the endotoxin standards are reacting as expected; that is, in accordance with the labeled, pre-calibrated sensitivity (λ, lambda). Also, a negative water control is only run to show that water used for sample or reagent preparation is not contaminated with endotoxin, an unlikely event.

Following the BET gel-clot chapter literally would require the use of USP Endotoxin RS for preparation of positive controls. In practice, however, the industry uses more economical CSE preparations that are carefully made by the LAL reagent supplier and certified by appropriate documentation. The analyst must show that a CSE will reproduce the pre-calibrated, labeled sensitivity of the LAL reagent when prepared according to the vendor's instructions and certification documents.

**BET Photometric Techniques**

The determination of compliance with a photometric method is made by looking at the language of *USP* Chapter <85>, actual practice in the industry and reference to the vendor's instructions (package insert). The critical elements

for photometric techniques include: a validated method for preparing a sample, a PPC to check for interfering conditions, selection of non-interfering test materials, generation of a standard curve that meets specific criteria and use of an incubating spectrophotometer.

There are aspects related to photometric techniques that wisely were not specifically addressed by the BET authors. For example, the BET chapter does not specify glass versus plastic, the exact number of concentrations for the standard curves, the range for predetermined absorbance (onset optical density) levels, wavelength for the photometer, incubation times or the method for regression analysis for the standard curve. A BET analyst must rely on impeccable training, in-house practices and a good working relationship with their LAL reagent vendor to develop robust techniques for photometric BET assays.

A significant challenge for the LAL user or BET lab is the development of procedures that overcome the absence of pre-calibration data for kinetic LAL reagents. Without a point of reference for relating CSE standard values to the USP Endotoxin RS, such as in gel-clot methods, the analyst faces a finite uncertainty about the values generated by liquid kinetic reagents. If CSE standards are too strong, endotoxin values in samples will be underestimated. If standards are too weak, values will be overestimated at the risk of OOS events. A BET laboratory is dependent on the skill of the BET analysts, robust procedures and the quality of LAL reagents to generate accurate and reliable test results.

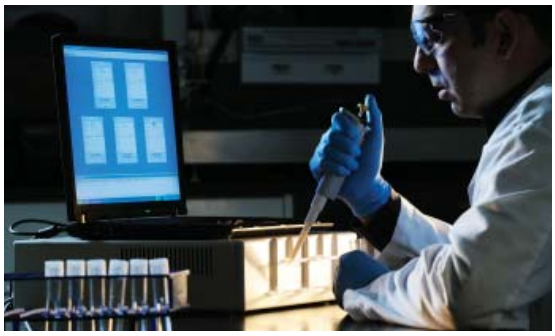
**LAL Cartridge Compliance with Photometric BET**

The LAL cartridge Portable Test System (PTS™) contains the licensed chromogenic LAL reagent that has been manufactured and released to specifications approved by the FDA. Therefore, the LAL cartridge method is described in the BET under kinetic chromogenic technique wherein a chromophore is released from a chromogenic peptide by the LAL/endotoxin reaction.

A significant feature of the LAL cartridge is the use of an archived standard curve that provides pre-calibrated reference to the RSE (see *Advantages of Archived Standard Curves for Endotoxin Measurement* in this issue). Variability associated with standard curves generated with liquid reagents and incubating microplate or tube reading spectrophotometers is eliminated.

The archived standard curve for the LAL cartridge is prepared using five cartridges for each concentration. That preparation means ten replicates for each concentration in the standard curve, which exceeds the minimum requirement. The criteria used for the correlation coefficient for the LAL cartridge curve also exceeds the minimum requirement, being set at greater or equal to 0.990. Therefore, the LAL cartridge meets or exceeds all BET requirements. In addition to BET compliance, each lot of cartridges is challenged with known concentrations of RSE (15 replicates at the high, middle and low range of the archived curve) to ensure accurate prediction before submission to the FDA for batch release.

For the end user to verify the LAL cartridge standard curve performance, the recovery of positive controls in the LAL cartridges is all that is required.



This step verifies the pre-calibrated sensitivity of the archived curve and confirms recovery of the positive control, as certified. In conformance with BET photometric tests, the positive control concentration is produced at the mid-point of the archived standard curve.

Since the PTS™ system does not require water for BET to prepare liquid reagents, the principal reason for the negative control is eliminated. In the LAL cartridge system, the sample under examination is itself used to rehydrate the reagents, and the PPC is pre-calibrated and certified. Therefore, water for BET may only be required for sample dilution. If necessary, the water for BET can be qualified at the beginning of use; absence of negative controls from the LAL cartridge system is not a risk to BET compliance.

**Conclusion**

The BET chapter is powerful because it is comprehensive without being a prescriptive document. Compendial bodies had no desire to stifle innovation, as evidenced by the introduction and adoption of quantitative methods from the mid 1980s. The Endosafe®-PTS™/MCS LAL cartridge systems are clearly aligned with the harmonized BET chapter. This new technology fulfills all requirements for a pre-calibrated test method, while at the same time delivers speedy results and simplicity of use in an innovative manner.

**References**


1. Cooper J.F. 2008 Bacterial endotoxins test, chapter 22. In: Prince R, ed. *Microbiology in Pharmaceutical Manufacturing, Vol. 2, 2nd Edition*, PDA, Bethesda, MD.
2. Williams K.L. 2007 Endotoxin as a standard, chapter 9, *Endotoxins, 3rd Edition*, Informa Healthcare, New York.
3. Hochstein H.D., Fitzgerald E.A. & Vargas R. 1994. Properties of US standard endotoxin standard (EC-5) in human male volunteers. *J. Endotoxin Res*. 1:52-56.

# LABORATORY NOTEBOOK

## Advantages of Archived Standard Curves for Endotoxin Measurement

*Masakazu Tsuchiya, Ph.D., Senior Research Scientist, Charles River*

An archived standard curve is a critical component of the Charles River Endosafe®-PTS™ (Portable Test System). The *Limulus* amebocyte lysate (LAL) cartridge was approved for use in the PTS™ by the U.S. Food and Drug Administration (FDA) in 2006, after careful review. However, archived standard curves are not new to LAL technology. Early generation tube readers for turbidimetric LAL methods had the capacity for archived standard curve methods, and the LAL test guideline of 1987 briefly addressed its use in photometric methods.

Given this historical perspective, why did the archived standard curve diminish in importance until just recently? One reason is the recent two-decade dominance of LAL technology by kinetic microplate readers for chromogenic and turbidimetric assays. The rate of the endotoxin-LAL reaction has marked heat dependency. Therefore, the uneven heat distribution of early-generation microplate readers and poor heat transfer properties of polystyrene microplates introduced so much variation that archived standard curve applications became impractical.

The onset times of endotoxin standard dilutions are also affected by the start time of the measurement that depends on the sample number and the techniques of the operator. However, the precise temperature control in the PTS™ unit and the prompt, simultaneous reactions in the LAL cartridge create ideal conditions for an archived standard curve. The harmonized Bacterial Endotoxins Test (BET) was written during the period that archived standard curve applications had fallen out of favor, which is why it was simply not addressed. As indicated in this issue, the PTS™ system clearly meets the criteria for generating photometric standard curves.

The greatest advantage of using the PTS™ is that it contains a pre-calibrated system that is analogous to the pre-calibrated sensitivity certification (label claim) that vendors provide for gel-clot LAL reagents. In both cases, the end user is asked to confirm that the positive control is recovered as certified. For example, the archived standard curve is verified when the end user reclaims the positive controls that are immobilized in the LAL cartridges. This pre-calibration enables end users to conduct LAL measurements with the same skill and dependability as the vendor.

This article provides data that supports the advantages of an archived standard curve of the PTS™. For this purpose, one lot of Positive Sample (PS) was prepared for this experiment. PS was comprised of a U.S. Reference Standard Endotoxin dilution with stabilizers, mannitol and sodium chloride, in single-test vials. The vial-to-vial reproducibility was 4.2%-6.3% as analyzed by the kinetic turbidimetric and kinetic chromogenic assay methods, and the potency was approximately 0.2-0.3 EU/vial. Analysts could use PS by reconstituting a vial with 1ml LAL Reagent Water (LRW). Since the reconstituted PS was used without further dilutions, the bias from the preparation of PS was minimal in this experiment. One vial of PS was reconstituted every day for this experiment.

Daily endotoxin standard curves were prepared with Endotoxin Standard for the Japanese Pharmacopoeia (JPSE) at 0.5, 0.1 and 0.02 EU/mL (by using PTS™). Potency of the reconstituted PS was calculated by using the daily standard curve and was compared with the potency of the archived standard curve established by the manufacturer.

**Table 1** shows the results of 16 independent assays. Averages of the potency of the reconstituted PS were 0.210 with the archived standard curve and 0.250 with the daily standard curves. Assay No. 3 shows a high value with the daily standard curve. The activity of the standard endotoxin dilution at 0.5 EU/mL was 71% of the average of 16 assays of 0.5 EU/mL dilutions. The PS for the No. 3 assay may have had slightly higher potency than the average because the PS showed 148% potency with the archived standard curve.

Plotting the 16 independent assays (Figure 1) also visually demonstrates that the potency of PS for assay No. 3 was higher than others. Considering the vial-to-vial reproducibility, the percent potency of 207% with the daily standard curve was too high. A combination of lower daily standard dilutions and a higher potency PS probably caused the high potency result in assay No. 3. The regular measurement method for

PTS™ using the archived standard curve did not show such unusual values, indicating that the archived standard curve method is more tolerant of randomly occurring irregular endotoxin standard potency changes.

When assay No. 3 was eliminated, the mean ASC/DSC in Table 1 was 93%. The bias of the measurement with the lot of PTS™ is about 10% against the JPSE vials used. Three vials of JPSE were used for this experiment, and the biases of the JPSE vials were slightly different between vials. The bias of a standard used in an endotoxin assay is not normally known when the assay is performed.

The bias of the measurement with an archived standard curve is caused by the endotoxin standard dilutions for the establishment of the archived standard curve, and is constant within a cartridge lot. The bias of the measurement with a conventional kinetic standard curve will be variable

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## RESULTS OF 16 INDEPENDENT ASSAYS OF POSITIVE SAMPLES WITH THE ARCHIVED STANDARD CURVE AND DAILY STANDARD CURVES

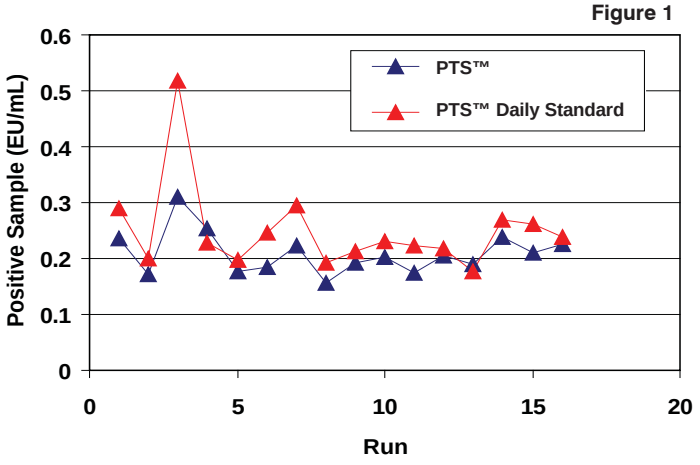
**Table 1**

| No | Archived Standard Curve | | Daily Standard Curve | | ASC/DSC | Mean ASC/DSC | JPSE |
|---|---|---|---|---|---|---|---|
| | PS (EU/mL) | PS% | PS (EU/mL) | PS% | | | |
| 1 | 0.236 | 113% | 0.289 | 116% | 82% | 85% | Vial# 1 |
| 2 | 0.171 | 82% | 0.199 | 80% | 86% | | |
| 3 | 0.311 | 148% | **0.517** | **207%** | 60% | | |
| 4 | 0.254 | 121% | 0.228 | 91% | 111% | | |
| 5 | 0.177 | 84% | 0.198 | 79% | 89% | 80% | Vial# 2 |
| 6 | 0.184 | 88% | 0.245 | 98% | 75% | | |
| 7 | 0.223 | 106% | 0.296 | 118% | 75% | | |
| 8 | 0.156 | 74% | 0.192 | 77% | 81% | | |
| 9 | 0.193 | 92% | 0.213 | 85% | 91% | 90% | Vial# 3 |
| 10 | 0.203 | 97% | 0.232 | 93% | 88% | | |
| 11 | 0.175 | 84% | 0.223 | 89% | 78% | | |
| 12 | 0.206 | 98% | 0.218 | 87% | 94% | | |
| 13 | 0.190 | 91% | 0.177 | 71% | 107% | | |
| 14 | 0.239 | 114% | 0.270 | 108% | 89% | | |
| 15 | 0.210 | 100% | 0.261 | 104% | 80% | | |
| 16 | 0.225 | 107% | 0.239 | 96% | 94% | | |
| Mean | 0.210 | 100% | 0.250 | 100% | 86% | | |
| SD | 0.038 | 0.184 | 0.079 | 0.316 | 12% | | |
| CV | 18% | 18% | 32% | 32% | 14% | | |

*Results of 16 independent assays of Positive Samples with the archived standard curve and daily standard curves.*

- *Mean ASC/DSC for assays numbers 1, 2 and 4 was 93%.*
- *Mean potency of the Positive Samples, excluding assay number 3, was 0.232 EU/mL.*
- *PS%: Percent potency of Positive Sample when the average of the 16 assays was set as 100%.*
- *ASC/DSC: Ratio of the potency of PS with the archived standard curve and that with the daily standard curve.*

**Figure 1**



## LABORATORY NOTEBOOK

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because it depends on the potency of the endotoxin standard dilutions and the skill of analysts. The degree of bias is difficult to determine, but trend analysis may be a useful tool. However, the archived standard curve method is probably more reliable than the daily standard curve method even if the assay procedures are controlled.

Microplate readers are unsuitable for creating archived standard curves because the starting timing of the measurement is not consistent across the microplate. The Charles River PTS™ is an ideal tool for the archived standard curve methods because the timing of the sample/reagent preparation and mixing is controlled by the PTS™ reader and the temperature control is impeccable.

Pre-calibration is central to the veracity of the archived standard curves in the PTS™ system. These curves are established by the LAL manufacturer using skilled and experienced operators for the LAL test. The manufacturer's QC laboratory is trained to recognize an irregular endotoxin standard potency change

and eliminate that variable. In other words, overall reliability of the PTS™ is very high because of the reliability of archived standard curves.

In conclusion, the results support the advantages of the archived standard curve method with the PTS™. A vendor-certified archived standard method is more reliable than traditional kinetic LAL methods, considering the risk of the inadvertent errors in the preparation of the endotoxin standard dilutions. The LAL cartridge creates ideal conditions for using an archived standard curve.

**A vendor-certified archived standard method is more reliable than traditional kinetic LAL methods, considering the risk of the inadvertent errors in the preparation of the endotoxin standard dilutions.**

## WHAT'S NEW

### FDA Publishes Rules and Guidance for PET Drugs

A Final Rule providing regulations on cGMP for Positron Emission Tomography (PET) drugs was published December 10, 2009. The rule applies to approved PET drugs, such as Fludeoxyglucose (FDG) F 18 Injection, USP. A guidance entitled, "PET Drugs – Current Good Manufacturing Practice (CGMP)" was also released to help producers better understand the U.S. Food and Drug Administration's (FDA) thinking about compliance issues. The Bacterial Endotoxins Test (BET) is a required release test.

### Publication on Automated Endotoxin Testing at Duke Medical Center

Faced with a need to rapidly determine endotoxin levels in batches of compounded sterile preparations (CSPs), the Duke Compounding Facility, under the direction of Kenneth Latta, adopted the LAL cartridge method for release testing for high-risk level CSPs. Validation data and test procedures were detailed in the publication. Citation: Cooper J.F., Latta K.S., & Smith D. Automated endotoxin testing program for high-risk level compounded sterile preparations at an institutional compounding pharmacy. *Am. J. Health-Systems Pharm*. **67**, 280-6 (2010).

## LAL POINTERS

The preparatory testing, as described in the *USP* <85> BET, is similar to the analyst, reagent and laboratory qualification that was initially detailed in the U.S. Food and Drug Administration's (FDA) LAL test guideline. Under the preparatory testing for the photometric techniques section, the *USP* states:

> *Using the Standard Endotoxin Solution, prepare at least three endotoxin concentrations to generate the standard curve. Perform the test using at least three replicates of each standard endotoxin concentration according to the manufacturer's instruction for the LAL Reagent.*

The validity criteria are defined in the *USP* in that "the absolute value of the correlation coefficient, |r|, must be greater than or equal to 0.980." The archived standard curve for the LAL cartridge system more than meets these criteria. The number of replicates used for each standard is 10, and the absolute value of the correlation coefficient, |r|, has to be 0.990 or greater.

Using the cartridge testing systems no longer requires that the analyst be proficient at preparing standard curves. Therefore, the initial qualification listed in the FDA-approved reagent insert references the initial qualification testing designed for LAL cartridge testing. The insert states:

> *Each new lot of cartridges must be qualified upon receipt. The initial qualification testing requires one cartridge with LAL reagent water as a sample. The evaluation must demonstrate no detectable endotoxin and acceptable spike recovery (50%-200%).*

In other words, to run the LAL assay using the LAL cartridge an analyst must simply be able to pipette accurately without adding contamination.

### Test for Endotoxin Down to .005EU/mL in Less than 20 Minutes

Endosafe®-PTS™ cartridges are now available in .005 EU/mL sensitivity with test results in less than 20 minutes. The FDA-licensed cartridges are pre-loaded with all of the reagents required to perform an LAL test with the Endosafe®-PTS™ portable spectrophotometer. When necessary, the .005 cartridges allow for a higher maximum valid dilution, increasing the likelihood of overcoming product interference. Quantitative results are displayed on the screen and can be downloaded to a central data management system.

### Detect Glucan Contamination in Less than 30 Minutes

Endosafe®-PTS™ Glucan Assay is a rapid test designed for investigational purposes to validate that your products are free of (1,3)-ß-D glucans, which are molecules found in the cell walls of most yeasts and molds. Glucans contaminate cell culture fluid, yeast protein production, air quality samples and cellulose filter preparations, causing false-positive results in LAL assays and triggering OOS investigations. The glucan cartridges have a sensitivity range of 10-1,000 pg/mL, yield results in less than 30 minutes and can be run on the same Charles River PTS™ reader that is used for endotoxin detection and Gram identification.

## UPCOMING SEMINARS & WORKSHOPS

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

- April 14-15
  *Italy*
- April 27-28
  *Germany*
- June 8-11
  *France*
- June 10
  *Puerto Rico*
- August 24-27
  *South Carolina, USA*
- September 15-17
  *England*
- September 21-22
  *Germany*
- September 28-October 1
  *France*
- November 30-December 3
  *France*

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