



Editor's Comments:

*Many complex and interrelated decisions must be made before kinetic LAL methods are implemented. This discussion provides guidance for this decision-making process. One is encouraged to anticipate actual needs and design the system accordingly. Advice and cautions are given for issues such as sensitivity, range and cost. Hopefully, this discussion will help avoid **the most expensive LAL test: the test that is repeated and investigated because of invalidity associated with sub-optimal test conditions.***

The omission of CSE from the harmonized BET has sparked lively debate. My review of USP documents indicates that no change in the use of CSE is required of LAL users.

Dr. J. F. Cooper

Starting a Kinetic LAL System

James F. Cooper and Foster T. Jordan

Pharmaceutical and medical device industries continue to move from gel clot to kinetic LAL methods in order to improve management of LAL-test data. However, many BET labs are unprepared to make the critical decisions that are necessary to implement kinetic LAL testing in a way that will meet the expected objectives. This discussion identifies and advises on critical choices that must be made before procedures are finalized. When informed choices are made at the outset, there is greater assurance that a kinetic system will perform as needed and will be initiated smoothly.

Table 1. Kinetic LAL Implementation Issues

Sensitivity	Robustness
Range	Cost
Speed	Reagent

Six of the most important issues that must be addressed before starting a kinetic LAL program are listed in Table 1. Most of the issues interrelate and must be considered collectively. It may be helpful to rank them by priority to gain a better perspective. One would be ready to select the reagent after considering the other five issues, *i.e.*, sensitivity, range, speed, robustness and cost.

SENSITIVITY

Two components determine the sensitivity of a BET. For infusion solutions and device extracts, the lowest point on the standard curve (lambda for kinetic LAL) and the amount of dilution determine sensitivity. For products that have an endotoxin limit in EU/mg, the choice of lambda and the concentration of the test material determine sensitivity. (1) The formula for product specific sensitivity (PSS) is a convenient way to calculate the sensitivity of a BET for this type of product, where

$$\text{PSS} = \frac{\text{Lambda (EU/mL)}}{\text{Test concentration (mg/mL)}}$$

The test concentrations used for current gel-clot tests can be substituted into the formula to confirm that a specific lambda provides adequate sensitivity; divide the new lambda by the validated (gel-clot) test concentration. It is critical not to adopt overly conservative endotoxin limits, because it requires a greater sensitivity. It should not be increased to the greatest extent arbitrarily; there should be a compelling reason, such as troublesome inhibition or revised testing to cover intraspinal administration. The greater the sensitivity, the greater is the risk of invalidity and hot-wells; a discretionary increase may be detrimental to good BET-lab management. A requirement for greater sensitivity (<0.01 EU/mL) favors the choice of chromogenic LAL because it gives a better signal-to-noise ratio, which in turn minimizes the incidence of hot wells.

STANDARD CURVE RANGE

When kinetic LAL for microplate readers was promoted a decade ago, LAL users were encouraged to adopt wide-range standard curves with five points covering a 4-log range of 50-to-0.005 EU/mL. We have learned that this range is not in the best interest of BET labs. Early use of kinetic methods was fraught with a high incidence of test invalidity, usually enhancement artifacts, where the PPC was not recovered within the allowable limits. The principal cause was use of a wide-range curve that exceeded the sensitivity of the LAL reagent and stability of the endotoxin standard. Also, the 50 EU/mL standard reacted so quickly that it became problematic if a full microplate was prepared. A two or three log range is now the choice of most experienced labs.

There is merit in selecting one standard range and simplifying protocols in a lab that conducts only one type of testing. However, if there are different kinds of tests being done, it is advisable to consider the best standard curve for the application. For example, if a lab's duties include water testing, product and raw material testing, monitoring of a recombinant's endotoxin levels, and support for equipment validation, it

may be prudent to have two or more standard ranges and templates that best fit the task. With newer software, such as Endoscan-V, storage and security of multiple templates is less problematic than before.

SPEED

The time that is required for a kinetic system to detect the onset times for the lowest points on the standard curve is an indicator of the speediness of a test. Lambda and the onset optical density (OD), an instrument setting, influence speed. The onset OD determines the threshold for change in optical density that is required to signal a positive reaction. If turn-around time were a major factor in ranking priorities, then one would select an intermediate standard range and a low onset optical density to increase the number of studies without investing in an additional microplate reader. Low onset OD settings may be associated with an increased risk of hot wells.

ROBUSTNESS

In the BET lab, robustness means that LAL testing is accomplished with a paucity of invalid results, hot wells or occurrence of out-of-specification (OOS) results. It is influenced by the choice of the three Rs, range, reagent and regression analysis. A two-log range of 5-to-0.05 EU/mL is the most robust of all standard curves. Kinetic chromogenic reagents are advantageous for curves where lambda is ≤ 0.01 EU/mL because the signal is more robust. The choice of regression analysis also has an impact on robustness. Polynomial regression was introduced to minimize invalidity problems associated with wide-range standard curves. Unfortunately, this regression conceals poorly performing reagents and loss of potency in the lowest endotoxin concentrations. These factors lead to background or plate noise being recorded as hot wells or “false-positives,” which in turn prompt needless OOS investigations. However, if one applies the tight linearity requirements (see regulatory section below) before entering polynomial analysis, determined endotoxin values may have greater accuracy.

The care taken in validating a LAL method for a product impacts on robustness. If products were first validated at a concentration or dilution near the interference level, it is meaningful to use revalidation with a kinetic LAL method as an opportunity to identify a more suitable, robust test concentration, as previously discussed. (2)

COST

Although the gap in price between KCA (kinetic chromogenic analysis) and KTA (kinetic turbidimetric analysis) has decreased, the expense of the chromogenic substrate in the KCA reagent will always be a factor. For mid-range standard curves, there is no significant advantage of KCA over KTA, and KTA is clearly the best choice for economic reasons. For wider and more sensitive kinetic ranges, KCA has a slight edge because of speed and a more robust signal. The greatest economy is in selection of a robust system that performs consistently with a minimum of invalidity. With good technique and quality accessories, Endosafe KTA² performs well in the ≤ 0.01 EU/mL range.

REAGENT

The reagent can be selected after the parameters discussed above have been carefully considered. If a wide-range standard curve or great sensitivity is a high priority for a BET lab, the KCA method would be favored because it provides greater robustness under these circumstances. For the same benefit of robustness, KCA is the reagent of choice if cost is not a factor. If a medium range standard curve is preferred, and if great speed is not an issue, the KTA method would be the reagent of choice because it yields high performance with the lowest cost.

For BET labs that struggle with the issue of LAL Reactive Glucan, the choice of reagent for specificity depends on the objective of the test. To exclude LRG as a contaminant, such as in filter washes, one would select an LRG-responsive reagent to identify its presence. However, to test a product that contains glucans, such as carboxymethylcellulose, one would select an endotoxin-specific method.

REGULATORY REQUIREMENTS

Requirements for a harmonized kinetic BET include: 1) three or more standard points, 2) linearity [r] of -0.980 or better, 3) recovery of 50-200% for positive controls and 4) an incubation temperature advised by the supplier. These criteria are woefully inadequate minimum criteria for kinetic LAL. The overly permissive [r] requirement is misleading because recovery criteria are seldom met if [r] values are below -0.99 . Linearity should consistently be ≥ -0.997 for a 2-log standard curve and ≥ -0.995 for a 3-log curve (4 points). If these goals are not met, conditions for optimizing linearity should be pursued. Further, it is critical to limit variability by setting a suitable coefficient of variation, $\leq 10\%$, so that widely divergent results in 2 wells can be assigned as invalid rather than OOS. Ideal instrument settings and analysis criteria listed in Table 2 can minimize invalidity.

Table 2. Ideal Analysis and Instrument Settings for Kinetic BET Methods

Standard Curve Range	2-3 logs
Lambda	≥ 0.01 EU/mL
Standard Interval	10-fold
Onset OD	≥ 0.03 units (30 mOD)
Regression Analysis	Linear
Analysis Time	< 60 min
Coefficient of Variation	$\leq 10\%$

In summary, validity can be assured by careful selection of the LAL reagent, instrument settings, kinetic reader and standard curve range at the time of system introduction.

References:

1. Cooper JF. BET calculations for multiple component parenterals. *LAL Times*, Vol. 5, No. 3, 1998.
2. Cooper, JF. Validation of bacterial endotoxins test methods. *LAL Times*, Vol. 6, No. 2, 1999.
3. Cooper JF, ME Weary & FT Jordan. The impact of non-endotoxin LAL-reactive materials on LAL analyses. *PDA J of Pharm Sci Technol* 51:2-6, 1997.

The Role of Control Standard Endotoxin (CSE) in Routine BET Testing J. Cooper
(The new harmonized BET of the USP was reviewed in *LAL Times*, March 2000.)

The LAL community has expressed concern that failure to address the CSE issue in the new BET represents a change in USP policy toward the RSE/CSE ratio determination and use of CSE in routine BET testing. Further review of this issue indicates that no change in policy by USP was intended regarding CSE.

The BET text did not contain instructions for RSE/CSE determination because each pharmacopoeia will designate its own reference standard. In the spirit of harmonization, the USP elected to resolve the CSE issue by other means so that implementation would not be delayed.

The preamble to the new BET that appeared in the January 2000 **Pharmacopoeial Forum (PF)** stated that CSE was deleted because in-house standards (such as CSE) are known to be equivalent. Further, in <11> *USP Reference Standards*, it states that the suitability of non-referenced materials rests with the purchaser. From these statements in USP documents, a BET lab may conclude that confirmation of label claim of a certified RSE/CSE ratio is an appropriate test for suitability for a CSE supplied by a LAL producer.

In summary, one may conclude that the omission of the RSE/CSE determination from the BET means that the USP considers the use of CSE to be an established practice that does not require elaboration in a harmonization text. LAL users should continue as before; a record of confirmation of label claim is sufficient evidence of CSE suitability. Nevertheless, it may be useful for the USP to incorporate an Informational Chapter into the compendia that describes how to demonstrate equivalence of CSE with USP Endotoxin RS. Until further action by the USP, it is recommended that one keep a copy of the January 2000 **PF** preamble statement as documentation of the USP's policy about equivalence of CSE to the reference standard. Otherwise, this statement, which is not included in the new BET chapter, may fade into obscurity with time.

The LAL community will continue to have carefully calibrated CSE available from LAL suppliers.

CHARLES RIVER ENDOSAFE'S POSITION: John Dubczak, Operations Manager

Endosafe will continue to supply customers with CSE. We will continue to calibrate our in-house CSE standards with every lot of LAL and with relevant LAL methods. The qualitative aspects of our CSE standards will not change. Endosafe will continue to supply CSE calibrated against USP Endotoxin RS according to procedures described in the original text of the BET in **USP 24**. A discussion of CSE certification procedures appeared in *LAL Times*, Vol. 5, December 1998.