LAL Update®

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Letter from the President

This issue of the UPDATE is the second in a three-part series related to Endotoxins — Facts and Fiction. Over the years there has been a lot of discussion of the standard curve as it relates to the turbidimetric and chromogenic assays. In this issue, I will compare various forms of the standard curve, methods for spike recovery, and sensitivity considerations. I hope a better understanding of the alternatives and reasons for their employment will allow the LAL user to make intelligent, practical choices.

As ACC continues to grow and starts the new year, I would like to introduce Mr. Carlos A. Castro, the newest member of our technical services department. Carlos, a native of Chile, speaks Spanish and English fluently. He has a B.A. degree from the University of Chile, Santiago, and an M.A. from the University of Rhode Island. Prior to joining ACC, Carlos worked for the National Marine Fisheries Service in Woods Hole. He is an accomplished speaker, writer, and workshop organizer who I expect will rapidly become a valued member of the ACC team.

The 1999 product catalogs for ACC and SAM are here! Please contact Debbie Fraser at (800) 848–3248, ext. 2223 or by email at dfraser@acciusa.com to request a copy. Be sure to include your complete mailing address and specify which catalog you would like to receive.

It also gives me great pleasure to announce the expansion of Associates of Cape Cod, Inc. through a merger with Seikagaku America, Inc. (SAM) of Ijamsville, Maryland. SAM is a provider of high quality research biochemicals and specializes in products related to glycobiology. As of January 1, 1999, all SAM activities will be run out of the ACC facility on Cape Cod and SAM's Maryland location will be closed. SAM will be treated as a division of ACC and as such will have its own catalog, sales personnel, and distributors. However, ACC customer and technical service as well as its shipping department will serve both ACC's and SAM's products. One change LAL customers will see is the addition of SAM's logo next to ACC's on shipping papers and invoices. Another change involves moving ACC's "research" products, e.g. the END-X® Endotoxin Removal Devices to the SAM catalog. Several new products that may be of interest to LAL users can also be found in the SAM catalog: a "research only" endotoxin-specific LAL, ENDOSPECY; and an Endotoxin Neutralizing Protein for use as a control in experiments involving the effects of endotoxin in cell culture or animal experiments. Anyone interested in these specific products or in fine biochemicals in general, should write or call for the SAM catalog. This catalog, as well as a new ACC catalog are now available.

Sincerely,

Thomas J. Novitsky, Ph.D.

Standard Curves: Long vs. Short; Curved vs. Straight — Which is better?

By Thomas J. Novitsky, Ph.D.

The arguments surrounding the range of the standard curve are outstanding examples of misunderstandings that can develop when marketing and science mix uncontrollably. Consider the following statements: The ability to construct a wide-range standard curve is indicative of a high-quality LAL; Wide range standards are better since a repeat standard will not be needed if a large amount of endotoxin is found in the sample; Four concentrations are just as good as six for constructing a standard and reagent is conserved; It is easier to recover a spike using a wide-range standard. I will also address the relative sensitivities of the turbidimetric and chromogenic methods.

Before discussing each of these points, it should be noted that they apply mainly to the kinetic LAL assays. In an end-point test, using either the turbidimetric or chromogenic reagent, it is unlikely that a standard can be constructed from a single, fixed incubation period that will span much more than a range of one logarithm. In fact, this was one of the reasons for the development of the kinetic assay. In order then to change the limits of detection in an end-point assay, the incubation time must be changed. This results in a "shift" of the detection range. Thus, a "sensitive" assay which detects between 0.006 and 0.1 EU/ml in 45 minutes, can be "made" less sensitive by decreasing the incubation time. For example, using this same reagent with an incubation time of 30 minutes might result in a linear standard in the range of 0.06 to 1.0 EU/ml. Once a convenient way was found to continually read the optical density (as turbidity or color) while incubating and timing the assay, the kinetic assay became feasible. Kinetics made possible an expanded range for the standard curve, at least up to the limits of substrate availability (in the chromogenic assay) or the ability of the reader to differentiate between high concentrations of standard where the reaction rates are extremely fast (in both chromogenic and turbidimetric assays). Thus the maximum range of the existing reagents is roughly 0.001 to 100 EU/ml.

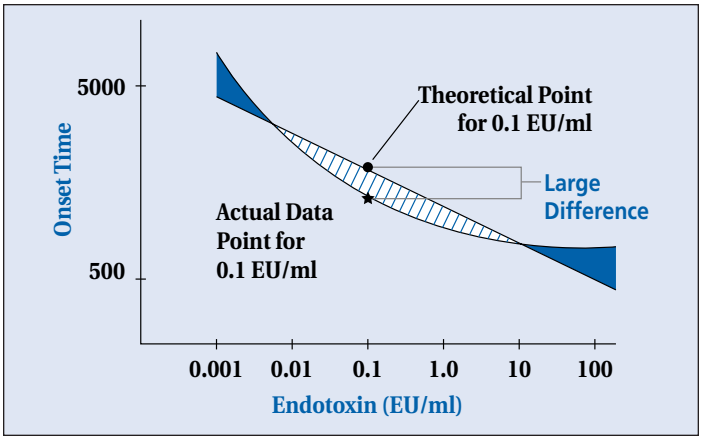
Does a wide range curve indicate a high quality LAL reagent?

With the introduction of the first kinetic LAL test utilizing Pyrotell-GT and the LAL-4000, it was found that although wide standard curves were possible, greater precision could be achieved if smaller ranges were used. This was because a wide range standard "line" usually was a "curve," i.e., not a straight line according to the equation Y=aX+b where: Y=log on set time; X=log endotoxin concentration; a=slop of the line; and b=the Y intercept. We know that often an LAL lot is produced that will yield very linear standard curves over a wide range of endotoxin concentrations. However, in other lots the curve may be more pronounced, even though in all respects the lot is perfectly acceptable for use. It is of course the multi-enzyme nature of the LAL reaction which results in complex kinetics, and the natural variability between raw LAL extracts (season, location, fitness of the horseshoe crab, etc.) that results in different characteristics for different LAL lots. Thus the statement that only high quality LAL can produce a wide range standard is false. The true statement is that all lots of LAL can produce a wide range standard curve, with some lots being better than others (as far as linearity is concerned).

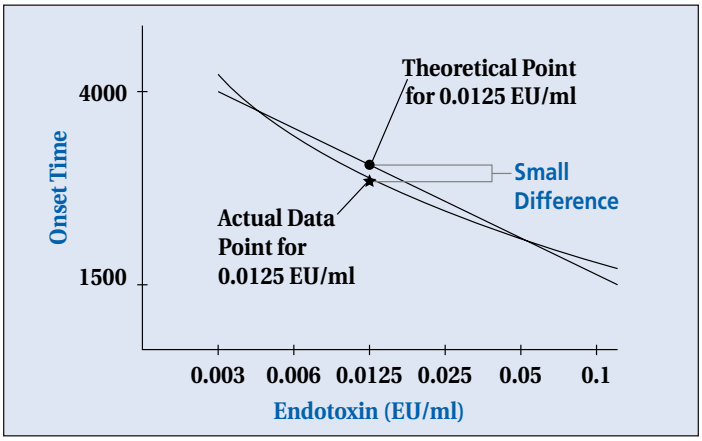
Are wide range standard curves better?

It might also seem that wide range standard curves are better simply because they cover all the bases. This is certainly true if a user expected a wide range of endotoxin concentrations to be encountered. When I was examining seawater samples during my oceanography days, I would have given anything for a kinetic LAL assay covering a range of 0.001 to 100 EU/ml. As it was, I had to test a series of dilutions of each sample to insure that at least one fell in the range of the standard curve of my end-point assays. In today's quality assurance environment however, the levels of endotoxin in many samples never exceed 0.25 EU/ml, let alone 1 or 10 EU/ml. Labs screening raw materials or monitoring water systems (where the feed water is also measured) will of course encounter higher concentrations. Therefore, it is necessary to consider the nature of the samples to be tested before deciding on which range of standards to use. If the lab limit on finished and in-process samples is 0.25 EU/ml, it seems a waste of time (and money for standard and LAL reagent) to run standards as low as 0.001 or as high as 10 EU/ml. A simple formula, like the one used with the gel-clot assay, would be to "bracket" the concentration of interest. Thus for a sample limit of 0.25 EU/ml, a standard series of 0.06, 0.125, 0.25, and 0.5 EU/ml would be suitable. A "spike" at 4λ ($\lambda=_{0.06}$), or 0.25 EU/ml would also be logical and easy to construct.

Keep in mind that dilution to overcome inhibition/enhancement also dilutes the endotoxin in the sample. Therefore, labs that test raw materials almost always dilute samples prior to testing. This not only removes sample interference, but also lowers the endotoxin concentration in the material to levels close to or lower than the standards selected. Even labs testing feed water to purification systems can encounter inhibition from metal ions and organic contaminants which necessitates dilution with LRW, thus lowering the endotoxin concentration. With careful thought, one standard curve of a smaller range could be used for most if not all samples. In addition to saving time preparing samples, the smaller curve



(Figure 1) Wide Range (0.001–100 EU/ml)



(Figure 2) Narrow Range Standard Curve (0.003–0.1 EU/ml)

provides added precision (use twofold rather than 10 fold dilutions) and linearity. Simply put, bigger is not always better!

Is it really easier to recover a spike using a wide range standard?

Once again, the linearity of the standard line is of paramount importance. If the standard line is curved to any appreciable degree, recovering a spike in the center of the standard line is more difficult (Figure 1). This is especially true for wide range standards. On the other hand, the use of a narrow range results in a closer approximation of a straight line. As an example, consider an arc (curved wide range standard line) bisected by a cord, (a straight line which bisects the arc at two points). As the cord, which represents a standard line, calculated via linear regression, becomes smaller, it more closely approximates the line of the arc (Figure 2-note the narrower range of endotoxin concentrations compared with Figure 1). Therefore, using linear regression to construct a standard line, it is actually easier to recover spikes from a narrower range of standards. This of course was one reason ACC originally recommended narrow standards covering a range of interest for its kinetic turbidimetric system. The problem can also be resolved for wide range standards by using a curve-fitting program rather than linear regression. Remember, however, that the FDA guidance requires that the LAL reagent must always meet a linearity requirement of $\geq |0.980|$, even though a curve-fitting formula is used for routine analysis. Apart from statistical considerations, small amounts of inhibition/enhancement can be "hidden" through the selection of the standard curve, and the spike concentration. Thus, the use of a tighter (i.e., narrower range) standard curve and selection of the spike as close to the product limit as possible should give the user more confidence in the quality of their products with respect to endotoxin contamination.

Which is more sensitive, the chromogenic or the turbidimetric assay?

This is a question which should probably be left to the marketing people to (continue to) fight over. The fact is, being an enzyme assay, the LAL test is subject to a variety of variables which affect the rate of reaction. Some of the more obvious ones are pH, temperature, and ions, while some of the less obvious are influence of the reaction vessel and (surprise!) the chromogenic substrate itself. If I wanted to make the fastest, i.e., most sensitive LAL test in the world, I would use 40°C rather than 37,° glass reaction vessels rather than plastic, a pH of 7.0, an ionic composition similar to seawater, and the native, i.e., coagulogen substrate. The later highlights an interesting point, that is all chromogenic substrates have less affinity for the *Limulus* clotting enzyme, i.e., are less reactive, than coagulogen. Chromogenic substrates also have pH optima for cleavage that are quite different from that for coagulogen (and from the activation of factor C). These latter two reasons are why the original chromogenic assays were two-step (see Roslansky and Novitsky and LAL UPDATE Vol. 1, No. 1 for a more detailed discussion). Aside from these reasons favoring the "natural" kinetic turbidimetric assay as the most sensitive, an end-point chromogenic assay with diazo-coupling can be similar in sensitivity. For those users truly interested in determining the "sensitivity" of their assay, or more importantly the "limit of detection" (LOD), and "limit of quantitation" (LOQ) rather than simply referring to the "λ" or "lowest standard used" definition, try applying the following equations:*

Limit of Detection (LOD) or the lowest "believable" measured value, one which is larger than the uncertainty associated with it.

$$LOD = 3s_{blank}$$

where s_{blank} is the value of the standard deviation of the negative control

Limit of Quantitation (LOQ) or the lowest level where measurements become quantitatively meaningful.

$$LOQ = 10s_{blank}$$

Always remember, if you can't "see" or measure the blank (negative control), these exercises are meaningless since the actual negative control may be within the LOD! It is important therefore to

"measure" the negative control (in extremely sensitive assays you should be skeptical of any negative controls that don't show some reaction). If the lowest standard is within 3 standard deviations of your blank, repeat the assay being careful to use the highest quality water while using extreme care to prevent inadvertent endotoxin contamination. If the blank is still too close to your lowest standard, you will need to reevaluate the sensitivity of your test. The good news is that the limits of 0.001 and 0.005 EU/ml used with the commercial LAL reagents available today are conservative, and, in practice, the LOD is significantly less than these values.

In conclusion, once the enzymatic nature of the LAL test is considered and proper statistical methods applied to carefully conducted assays, the user will be in a much better position to decide which of the available methodologies are best suited to his/her testing program.

***Note:** All these measurements require standard curves constructed with at least three concentrations replicated 7 times (n = 7) including the blank or negative control. In addition, the concentrations should be selected near the most sensitive part of the standard curve.*

Additional Reading

Quality Assurance of Chemical Measurements, John Keenan Taylor, Lewis Publishers, Inc. Chelsea, Michigan, USA. 1987.

Bacterial Endotoxins Test, p. 1696–1697. In USP 23 NF 18, 1995. U.S.Pharmacopeial Convention. Inc., Rockville, MD.

"Erratum" ***In the June 1998 LAL UPDATE, Vol. 16, No. 2, the ordinate axis on the graph in Figure 4b was mislabeled. The "0.6 " figure should read "3.0."***

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