LAL UPDATE®

Volume 13, No. 3 **September 1995**

Dear LAL User,

Another summer has ended on Cape Cod and with it another successful crab bleeding season. Although our summer was exceptionally hot and dry, the crabs stayed cool and moist in our specially designed facility until returning to the ocean after their blood "donation."

In addition to our normally hectic summer pace, Associates continued to expand its operation. We were exceptionally pleased when Dr. Jack Levin, co-discoverer of LAL, accepted a position on our Board of Directors. Dr. Levin's ties to Woods Hole extend back to the 1960's with his introduction to Limulus by the late Dr. Frederik Bang. During his Woods Hole summer research sojourns, Dr. Levin became acquainted with Associates' founder, the late Dr. Stanley Watson. Together they co-edited several proceedings of the Woods Hole conferences on Limulus and LAL. Dr. Levin brings to Associates of Cape Cod his exceptional expertise and understanding of LAL. In addition, his knowledge of medicine, especially endotoxemia, sepsis and infectious disease, will help us develop clinical applications for LAL.

I am also pleased to announce that Dr. Jack Sloyer has become our Director of Product Development. Dr. Sloyer comes to us with a solid background in clinical diagnostics and industrial microbiology and will be working on some new bacterial detection products. Jack is also no stranger to LAL, having published several papers in the field, including one of the early studies on turbidimetric LAL kinetics.

This LAL UPDATE contains a summary of LAL test methods by Dr. Michael Dawson. As you may have noticed, more of the articles in the LAL UPDATE are being written by my very capable colleagues, Dr. Michael Dawson (Vice President, Production) and Dr. Marilyn Gould (Vice President, Regulatory Affairs and Responsible Head). I am sure they would appreciate your comments, and, as always, entertain any questions you may have.

Sincerely,

Thomas J. Novitsky, Ph.D.
Editor

A Wealth of Options

Choosing an LAL Test Method

by Michael E. Dawson, Ph.D.

Selecting the appropriate method can be difficult, especially for someone new to the LAL test. This article describes the choices, starting from the fundamentals of the LAL/endotoxin reaction, and includes a table summarizing the differences between the methods.

There are three principal LAL test methods: gel-clot, turbidimetric and chromogenic. The essentials of all three are included in the original model for LAL clotting proposed by Levin (1979) (Fig. 1). Endotoxin activates the clotting enzyme which then cleaves a soluble substrate. The resulting insoluble clotting protein forms a gel-clot.

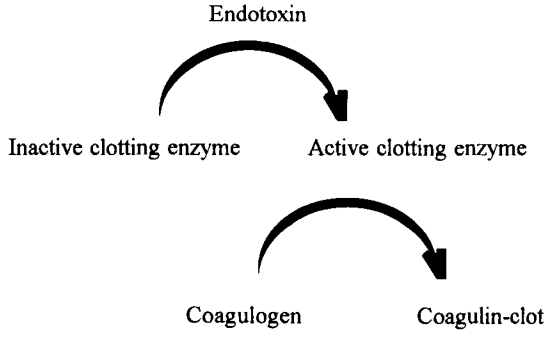

Fig. 1. The LAL Clotting Reaction (original model)

Since this model was first proposed, it has been demonstrated that activation of clotting enzyme is not direct. There are intermediate steps prior to the activation of the clotting enzyme. A more complex model, complete with the additional steps and an alternate pathway (Fig. 2), is now accepted. The intermediate steps amplify the response to endotoxin and help to give the LAL test its extraordinary sensitivity. Activated clotting enzyme cleaves a peptide from the inner portion of the coagulogen molecule. The two remaining peptides are linked by disulfide bridges to form

insoluble coagulin. Coagulin molecules associate to form a gel. The gel is fragile, particularly when it first forms. In contrast, when a horseshoe crab is injured in nature, the clot is apparently stabilized by the membranes of degranulated amebocytes and is quite resilient.

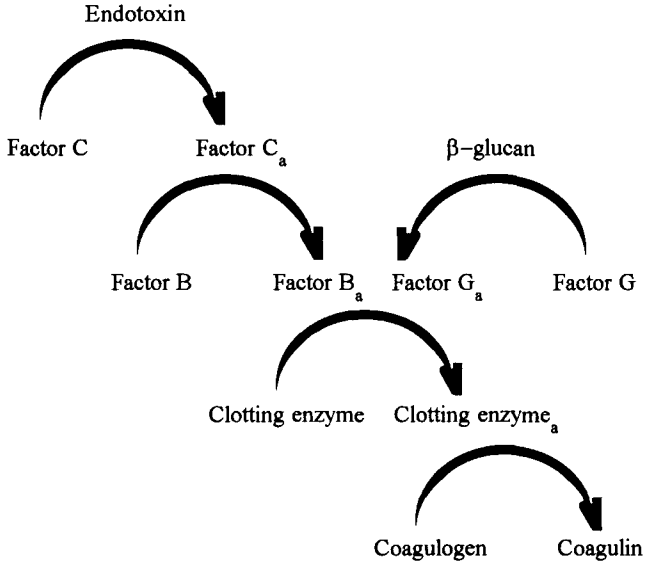

Fig. 2. LAL clotting cascade with alternate glucan activation pathway (Modified from Iwanaga 1985)

Gel-clot Method

The gel-clot method is the simplest and most widely used LAL test. The reaction in the test tube is essentially the same as that in nature when a horseshoe crab is injured. The gel-clot test is the compendial method. It is the only endotoxin test fully described in the United States Pharmacopeia (USP) and many other pharmacopeia, though other methods are permitted as alternatives.

Gel-clot reagent is labeled with a sensitivity (λ) which is the lowest endotoxin concentration to cause a clot to form under standard conditions. A series of twofold dilutions of Reference Standard Endotoxin (RSE) starting from 1 EU/ml is used to determine the sensitivity. Because the gel-clot test is a tube titration using twofold dilutions, the limit of resolution is one twofold dilution. The accepted error of the method is plus or minus one twofold dilution. Therefore, a reagent with a sensitivity of 0.125 EU/ml may not clot at this concentration in some tests. However, it should always clot at 0.25 EU/ml. Similarly, the reagent may clot at 0.125 EU/ml and at 0.06 EU/ml, but it should never clot at 0.03 EU/ml.

Because of the twofold error of the method, positive controls and positive product controls (spiked sample) are always at a concentration of 2λ (twice the labeled sensitivity).

The gel-clot test yields a binary result, either positive or negative. A tube is scored as positive (+) if the clot withstands 180° inversion without breaking. All other conditions are scored as negative (-), even if the clot almost remains intact but then collapses. This is not a subjective test despite some assertions to the contrary.

The test can be set up as a limits test to pass or fail a product at a particular endotoxin concentration. In this case, a negative result means that the sample contains less than the labeled sensitivity multiplied by the dilution factor of the sample. A positive result is reported as greater than or equal to the sensitivity times the dilution factor. Quantitation (or an assay) is achieved by testing a series of twofold dilutions of sample. The results of a typical gel-clot assay are presented in Table 1. The endpoint is the greatest dilution to give a positive test. The endotoxin concentration of the original sample is calculated by multiplying the reagent sensitivity by the dilution factor at the endpoint. By convention, results are reported as a specific value. In Table 1, the result is 2.0 EU/ml, not ≥2.0 EU/ml but <4.0 EU/ml. Because the error of the method is ± one twofold dilution, 2 EU/ml is not significantly different from 1 or 4 EU/ml.

TABLE 1. Example of gel-clot assay

Endotoxin Standard Concentration (EU/ml):

0.25	0.125	0.06	0.03	Neg. ctl.
+	+	-	-	-
+	+	-	-	-

Sample Diluted:

Undil.	1:2	1:4	1:8	1:16	1:32	1:64
+	+	+	+	+	-	-
+	+	+	+	+	-	-

Positive product control (undilute sample containing 2λ endotoxin)

\+
\+

LAL sensitivity (λ) = 0.125 EU/ml

In Table 1, the endpoint of the standard series is 0.125 EU/ml, which confirms the labelled sensitivity of the LAL. The endpoint for the sample is the 1:16 dilution; therefore, the endpoint dilution factor is 16. The endotoxin concentration in the sample is 16 x 0.125 EU/ml = 2 EU/ml.

Gel-clot reagent is available in a range of sensitivities. In order to decide which sensitivity to use, a number of factors must be considered. First, determine the endotoxin levels or limits to be detected. Clearly the reagent must be sufficiently sensitive to at least detect the limits. Also consider the type of sample and, if possible, the likelihood of it interfering with the test. A greater sensitivity will give increased scope for dilution to overcome interferences. More sensitive reagent is

recommended for blood products, many other biological materials, and for electrolyte solutions containing trace elements. Less sensitive reagent is commonly used for water, saline, and for many drug products. Ultimately, the sample must be tested to assure that it does not interfere with the test at a dilution at which the endotoxin limit can be detected. It may be necessary to try a more sensitive reagent if interference cannot be overcome. One approach is to always use a sensitive reagent. The disadvantage of this strategy is that greater sensitivity means more dilutions of samples and standards, which is unnecessary work and consumes supplies. The selected sensitivity ultimately depends upon the type of sample to be tested and upon personal preference. Associates of Cape Cod's technical representatives are always available and willing to discuss the issues involved and advise on the choice of a sensitivity.

Turbidimetric Methods

As the concentration of insoluble coagulin increases during the LAL reaction, the turbidity of the reaction mixture increases. The rate at which turbidity increases is related to the endotoxin concentration in the sample and is the basis of the methods. There are two variations of the turbidimetric method.

Endpoint Turbidimetric Method

After the sample and LAL reagent have incubated for a fixed period of time, the absorbance is read in a spectrophotometer at a wavelength less than 450 nm (405 nm or below is preferable). Higher endotoxin concentrations in samples or standards give greater absorbance. Standard curves are constructed by plotting the absorbance against the known endotoxin concentrations of standards. Because standard curves allow interpolation between the standard concentrations, the method has greater resolution than the gel-clot method. This is also true for the kinetic turbidimetric and chromogenic methods. The endpoint turbidimetric method requires that timing of incubation and reading be controlled carefully because development of turbidity can not be stopped. Consequently, each sample tube or well can only be read once. The range of endotoxin concentrations that can be detected is limited. Only those concentrations that are sufficient to cause development of measurable turbidity within the incubation time, but are not so high that the reaction has reached completion (saturation) may be quantified in a given assay. Concentrations below the minimum are indistinguishable from negative controls. At concentrations above the maximum, turbidity approaches saturation and it is not possible to differentiate between them. The range of concentrations that can be detected is generally limited to approximately a factor of ten (e.g. 0.01 - 1.0 EU/ml or 0.5 - 5 EU/ml) for a given incubation period. Longer incubations give a more sensitive test up to a limit of 0.001 EU/ml.

Kinetic Turbidimetric Method

In contrast with the endpoint turbidimetric method, in which a single reading is taken for each sample, in the kinetic turbidimetric method readings are taken throughout the test. This requires an optical reader which incubates the reaction mixture at 37°C and stores readings at regular intervals (typically every 10 seconds). The time taken to reach a specific optical density threshold (the onset time) is recorded. At higher endotoxin concentrations, the reaction is rapid and the onset time is relatively short. Standard curves are constructed by regressing the log of the onset time on the log of the concentration of standard endotoxin. As the optical density is read throughout the assay, not after a fixed incubation time, the detection range of this method is very wide, from 0.001 EU/ml up to 100 EU/ml. Standard curves may cover part or all of the range. Kinetic methods require sophisticated software to analyze the results. ***Pyros*** for Windows™ for the LAL-5000 (Associates of Cape Cod, Inc.) is very easy to use and provides data summaries and reports.

Chromogenic Methods

Chromogenic methods utilize a synthetic substrate which is added to the lysate. Pyrochrome™ (Associates of Cape Cod, Inc.) has the substrate co-lyophilized with the LAL reagent, which enables it to be used for all chromogenic methods. The substrate has an amino acid sequence that is homologous to one of the points of cleavage of the natural substrate, coagulogen. A terminal chromophore (*para* nitroaniline, *p*NA) is linked to this peptide. The intact substrate is colorless, whereas free *p*NA is yellow with a peak absorbance at 405 nm. When chromogenic LAL reagent reacts with endotoxin, the cascade is initiated and clotting enzyme is activated as in the other methods. The clotting enzyme acts on the synthetic substrate, liberating the chromophore which turns the reaction mixture yellow. The reaction proceeds more rapidly at higher endotoxin concentrations, so the rate of development of the yellow color is greater. There are three variations of the chromogenic test, two endpoint methods and a kinetic method.

Endpoint Chromogenic Methods

In the endpoint methods, the reaction is stopped after a specific incubation period by the addition of acid. The absorbance of the yellow color is measured in a microplate reader or spectrophotometer. The higher the endotoxin concentration in the sample, the greater the amount of *p*NA liberated at the end of the incubation and consequently, the more intense the yellow color. The ability to stop the reaction is a great advantage over the endpoint turbidimetric

method but all of the endpoint methods share the limitation of a detection range of approximately 1 log for a given incubation time. The maximum sensitivity is 0.005 EU/ml.

In a variation, the diazo endpoint method, liberated *p*NA is coupled to a diazo compound after stopping the reaction. This result is an intense magenta color with a greater absorbance at 540 nm compared with *p*NA at 405 nm. This assay may be used to test samples which absorb at 405 nm, avoiding interference due to color.

Standard curves are constructed and unknowns quantified as described for the endpoint turbidimetric method.

Kinetic Chromogenic Method

The kinetic chromogenic method is very similar to the kinetic turbidimetric method described above, except that the optical density is due to the yellow color of *p*NA rather than turbidity. The test is typically performed using an incubating microplate reader. The range of endotoxin concentrations that can be quantified is 0.005 - 50 EU/ml.

Correlations Between Methods

Results obtained for the same sample generally correlate well. When comparing results, it is important to remember the error associated with each method. The plus or minus twofold error of the gel-clot method has been noted, but it is often forgotten that there are errors associated with the other methods as well. If the criterion for spike recovery is +/- 50%, as in some cases for kinetic methods, this is the error of the method. Thus, results of 0.1 EU/ml and 0.5 EU/ml may not be significantly different; the actual concentration might be 0.3 EU/ml. If results appear to be substantially different by different methods, it is important to insure that interference is controlled for all methods.

While results should be similar, the degree of inhibition or enhancement caused by a given product frequently varies between methods. The dilution required to overcome interference may be quite different for different methods (Table 2).

TABLE 2. Dilutions required to overcome interference for two parenteral products using three LAL methods

	Penicillin (200,000 Units/ml)	Clindamycin (150 mg/ml)
Gel-clot	1:16	1:32
Endpoint Chromogenic	1:10	1:20
Kinetic Turbidimetric	1:200	1:100

The results in Table 2 suggest that the endpoint chromogenic test is the least sensitive to interference, but given the test errors, the differences are not significant. Generally, the gel-clot method shows the least interference.

The various methods have different sensitivities. Consequently, the maximum valid dilution (MVD) at which the endotoxin limit of a product can be detected is different for each method. Higher test sensitivity allows for a greater dilution to overcome interference.

TABLE 3. Maximum valid dilutions (MVD) of two drug products for three LAL methods

	Sensitivity (λ) (EU/ml)	Penicillin	Clindamycin
Gel-clot	0.03	640	2,784
Endpoint Chromogenic	0.005	4,000	17,400
Kinetic Turbidimetric	0.001	20,000	87,000

Most samples can be tested by any of the methods. However, for some samples, one method is preferable to the others. Determination of the preferable method is often a question of trial and error, but it is not necessary to test all sample types by all methods. If a product cannot be validated and tested by a particular method, another should be tried. The gel-clot is often the best method to start with when uncertain about which one to use. It is straightforward to perform, the results are easy to interpret, and it incorporates the principle of all methods. Also, if a product has been validated initially by gel-clot and later by another method, the gel-clot method is always available as a back-up method in the event of equipment failure. If you need help in the selection of a suitable test method, our technical service representatives at Associates of Cape Cod are always available to help

References

1. **Levin, J.** 1979. The reaction between bacterial endotoxin and amebocyte lysate, p. 131-146. *In* E. Cohen (ed.), Biomedical Applications of the Horseshoe Crab (Limulidae), Progress in Clinical and Biological Research, Vol.29. Alan R. Liss, Inc., New York.

2. **Iwanaga, S., T. Morita, T. Miyata and T. Nakamura.** 1985. Hemolymph coagulation system in *Limulus*. Microbiology:29-32.

Comparison of LAL Test Methods

	Gel-clot	Endpoint Turbidimetric	Kinetic Turbidimetric	Endpoint Chromogenic	Kinetic Chromogenic
Cost	Lowest equipment cost	Relatively inexpensive, widely available instrumentation	Moderate to expensive instrumentation	Relatively inexpensive, widely available instrumentation	Most expensive instrumentation
Sensitivity	Sensitive - up to 0.03 EU/ml. Sensitivity standardized by the manufacturer	Most sensitive (detection limit of 0.001 EU/ml)	Most sensitive (detection limit of 0.001 EU/ml in the LAL-5000)	More sensitive (detection limit of 0.005 EU/ml)	More sensitive (detection limit of 0.005 EU/ml)
Maximum test range	NA	1 log e.g. 0.1 -1 or 0.01 -0.1 EU/ml	0.001 - 100 EU/ml	1 log e.g. 0.1 -1 or 0.01 -0.1 EU/ml	0.005 - 50 EU/ml
Resolution	plus or minus one twofold	+/- 25%	+/- 25 or 50%*	+/-25%	+/- 25 or 50%*
Susceptibility to interference	Resilient - often less affected by interference than other methods	May show more interference than gel-clot, but greater sensitivity gives more scope for dilution to overcome it			
Timing	Must be on hand to read test after one hour	Critical - reaction must be timed carefully	Automated instrumentation handles timing	Critical - reaction must be timed carefully but can be stopped for reading - easier than end-point turbidimetric	Automated instrumentation handles timing
Reaction vessel	Soda lime glass culture tubes	Borosilicate glass culture tubes or microplates**	Borosilicate glass culture tubes (in LAL-5000) or microplate**	Microplate**, sometimes glass culture tubes	Microplate** - allows for quick, easy dilutions
Other Comments	USP compendial method. Results are easy to interpret. Can process many samples at a time.		LAL-5000: - very good temperature control - individually controlled timing for each well - samples can be added to a test in progress	Diazo option allows testing of samples that absorb at 405 nm	

*The resolution of kinetic methods depends on the spike recovery range used. The 1987 FDA "Guideline on Validation of the Limulus Amebocyte Lysate Test ...." specifies that spikes be recovered within +/-25%. This was increased to +/- 50% in the 1991 FDA "Interim Guidance for Human and Veterinary Drug Products and Biologicals: KINETIC LAL TECHNIQUES". This change did not apply to medical devices.

**Microplates cannot be practically depyrogenated by the user. Occasional contaminated wells ("hot wells") are to be expected if they are used. An appropriate source of relatively clean plates is necessary. Pyroplates are available from Associates of Cape Cod, Inc. and are provided with a certificate of analysis.

Syringes Inhibit CSE Activity

If you are having problems with the potency and stability of reconstituted Control Standard Endotoxin (CSE), the syringes used during reconstitution of the CSE may be the cause. While several customers had reported isolated problems with syringes, ACC was unable to reproduce them until a few months ago when our technicians experienced problems. Potencies were reduced by more than twofold and tended to decrease over time. Our problem was traced to syringes located in the middle of an otherwise good box. Subsequent work showed that potencies were more reproducible when CSE was reconstituted using glass pipets than when using these syringes.

Notice of this problem has been included with Certificates of Analysis to make customers aware of the issue. If you experience similar problems, we recommend that pipets be substituted for the syringe and needle to determine whether or not you should continue to use syringes. To reconstitute CSE with a pipet, either lift the CSE stopper just enough to break the vacuum or insert a needle through the stopper to break the vacuum. Then remove the stopper. Add LRW with a pipet and seal the vial with Parafilm "M"®. Follow the instructions in the CSE insert for the remainder of the reconstitution protocol.

If you experience different activities between vials of CSE reconstituted with a syringe and those reconstituted with a pipet, please contact our technical services department and inform them of the details. We would like to know the manufacturer and lot number of any suspect syringes to help us and the manufacturer(s) determine what substance(s) may be causing the problem. We would also like to know of any adverse experiences with RSE lot F (EC-5). Note that RSE lot G (EC-6) is not stoppered under vacuum and is usually reconstituted with a pipet.

If you have any questions regarding syringes or CSE potency, please contact our Technical Services Department at **800-848-3248**.

Calendar

Defense and Civil Institute of Environmental Medicine
Sepsis and Septic Complications
"Endotoxin and Sepsis:
A Multi-center Clinical Trial of SepTest"
by Paul A. Ketchum, L.S. Stotts, T.J. Novitsky, J. Parsonnet and AMCC Sepsis Investigators.
Toronto, Canada
October 12-13, 1995

*** Come visit us ***

at the PDA 1995 Annual Meeting
Hynes Convention Center, Boston, MA
Booth 310
November 13-15, 1995
Poster: "Water Determinations in Lyophilized Product Using Near Infrared Spectroscopy (NIRS)"
by Marilyn J. Gould, Ph.D.
and
Kevin J. Barry

The American Automobile Manufacturers Association
The Industrial Metalworking Environment: Assessment & Control
Poster: "Rapid Determination (60 seconds) of Bacterial Contamination in Industrial Fluids"
by Dr. Jack Sloyer
Hyatt Regency Dearborn, MI
November 13-16, 1995

The 4th Conference of the International Endotoxin Society
October 22-25, 1996 Nagoya, Japan