LAL Update®
ASSOCIATES OF CAPE COD, INCORPORATED

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# Letter From the Editor

*Dear LAL User,*

*This month's LAL Update focuses on an issue faced by many LAL users: interference with the LAL test caused by the product or material being tested. Inhibition and enhancement are discussed in detail, along with some of the common causes of interference. Perhaps most importantly, a number of techniques for overcoming interference problems are presented. Even though many substances interfere with the LAL test, in most cases the problem is easily overcome. However, there are those samples that prove more difficult. This Update aims to help with these situations. The article concludes by highlighting of our Technical Services personnel, who are always available to help. Also, if you cannot solve a testing problem in your laboratory, our Contract Test Service will be happy to develop a test method, and if desired, to validate it.*

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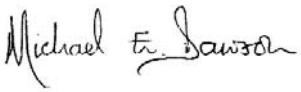
*With very best wishes for Happy Holidays and for a successful New Year,*

*Sincerely,*



*Michael E. Dawson, Ph.D.*

# Interference with the LAL Test and How to Address It

***By:*** ***Michael E. Dawson, Ph.D.***
***Director of Regulatory Affairs***

## Introduction

A majority of the substances tested for endotoxin interfere with the LAL test to some degree (the obvious exception being water samples). In a study reported more than 20 years ago, of 333 drug products tested, 236 (71%) interfered with the test prior to any dilution or treatment[1]. Fortunately, the LAL test is usually more sensitive than necessary to detect the endotoxin limit for a given product or material. It is often possible to overcome interference by diluting the sample to a point at which the interfering factor ceases to affect the test, but at which the endotoxin limit concentration is still detectable. The greatest dilution at which the endotoxin limit can be detected is the maximum valid dilution (MVD). (MVDs and their calculation are discussed in detail in a previous edition of the LAL Update[2].) Dilution is the simplest and most widely used technique for overcoming interference, and it is effective in the majority of cases. The MVD (i.e. the scope for dilution) can be increased by changing to a more sensitive LAL reagent or test method. Dilution should be tried before other methods of addressing interference.

Two classes of interference are considered here: inhibition and enhancement. Inhibition occurs when a material interferes with the ability of the LAL reagent to react with endotoxin, causing underestimation of the amount of endotoxin present. Tests must be properly controlled so that inhibition is detected if it occurs. Appropriate controls provide assurance that negative results are due to absence of endotoxin, not to inhibition. It is dangerous to test without controlling for inhibition. Failure to do so could result in release of a product on the strength of an invalid negative test result.

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Enhancement is interference that increases the sensitivity of the assay, resulting in overestimation of the endotoxin concentration of the sample. Enhancement is much less dangerous than inhibition. It could result in the inability to release a product that should have passed, but it does not result in a threat to public health and safety. It is therefore of less concern to regulatory authorities. The regulatory requirements for LAL testing reflect the reduced concern for enhancement. The positive product controls (PPCs) in routine gel-clot tests only control for inhibition, not enhancement. Routine tests by photometric methods (chromogenic and turbidimetric) show enhancement (and inhibition) clearly and unequivocally. However, the specification in the harmonized endotoxins test chapter in the United States, European and Japanese pharmacopeia (USP, EP and JP respectively) allow up to 200% enhancement, compared with 50% inhibition[3,4,5].

Two notes about enhancement: firstly, the phenomenon is sometimes referred to as a "false positive". This is not appropriate terminology because it is endotoxin causing the positive test result. It is recommended that the term be restricted to substances other than endotoxin that give a positive LAL test, such as (1→3)-ß-D-glucan and trypsin. Secondly, a positive, valid endotoxin test should be treated as contamination. "Enhancement" should never be used as a euphemism for contamination.

This article discusses various causes of interference and techniques by which it may be overcome. The task of overcoming interference is addressed during product characterization so that the test for interfering factors (called Inhibition and Enhancement Tests in the FDA Guideline on LAL testing[6]) may be conducted to validate the test. Product characterization was discussed in a previous LAL Update article under the name of "Preliminary Testing"[7]. Since the publication of that article, the harmonized pharmacopeial endotoxins test chapters have adopted this name to include LAL reagent performance verification and interference testing, so the term should only be used in that latter context.

Interference can result from either an effect of the sample upon the LAL reagent or an effect of the sample upon the endotoxin, notably its aggregation state and availability to participate in the reaction. An understanding of the mechanisms of interference can sometimes help the development of a strategy by which it can be overcome.

## Interference: Causes and Solutions

### pH

The LAL reaction consists of a series of enzymatic reactions in which serine proteases, each with a pH optimum, cleave their respective substrates. Consequently, it is critical that the pH of the reaction mixture of product and LAL reagent be in the range specified in the product insert. The harmonized pharmacopeial endotoxins test chapters require this, but go on to say that this is usually the case when the pH of the product (not the reaction mixture) is in the range of 6.0 - 8.0. However, it is quite possible that the pH of the product alone will be outside the 6.0 - 8.0 range, but that the pH of the reaction mixture will be within it because of the buffering capacity of the LAL reagent. This is commonly the case for water. In other cases, the pH of the reaction mixture may be outside the specified range for undiluted product, but the pH of a dilution of product (not to exceed the MVD) can be within the range. For example, if the pH of the reaction mixture is out of range for an undiluted product with an MVD of 1:200, but it meets specification at 1:20, then the product can be validated and tested at 1:40 or 1:50. There is no need to adjust the pH.

If pH problems are not overcome by dilution, use Pyrosol® buffer (or Glucashield® buffer if an endotoxin specific test is required) to reconstitute the LAL reagent as stipulated in the product insert and then check pH of the reaction mixture. (Pyrochrome® is the exception to this as it is supplied with a reconstitution buffer.) If necessary, the sample can be diluted with Pyrosol buffer and then tested. However, as using the buffer as diluent is not addressed in the product inserts, this should be considered a "treatment" and validated accordingly as described below. In samples for which the buffer does not resolve the problem, the pH of the sample (or of a sample dilution) can be adjusted by adding a solution of acid or base (HCl or NaOH). Prepare solutions from concentrated HCl or NaOH pellets with LAL reagent water (LRW). Before conducting any LAL tests, perform titrations to determine the appropriate volume and concentration of acid or base solution to bring the pH into range. The volume of added NaOH or HCl should not change the sample volume by more than 10%. If precipitation occurs, try diluting the sample first and then adjust the pH. If precipitation cannot be avoided, it may be necessary to test the sample with the precipitate, which may preclude the use of the photometric methods.

Finally, use of Pyrosol buffer will sometimes help to overcome interference even when the pH of the reaction mixture is within in the specified range. For interference problems that are not resolved by dilution, use of one of these buffers is the next strategy to try.

**Divalent Cations**

Divalent cations influence both the reactivity of endotoxin and the LAL reaction. Cations are attracted to and neutralize the negative charge of endotoxin, allowing increased aggregation size and decreasing activity/potency, which is observed as inhibition. Divalent cations can be expected to further increase aggregation by linking endotoxin subunits by cation bridging. Divalent cations are also required for optimal LAL sensitivity, but excess concentrations inhibit the reaction. Dilution is the usual solution to this problem. When interference by salts (or other small molecules) cannot be overcome by dilution, endotoxin can be separated from interfering substances by ultrafiltration, as discussed below.

**Chelating Agents**

Materials which bind (chelate) divalent cations, such as EDTA (ethylene diamine tetraacetic acid) and heparin, can reduce the aggregation state of endotoxin. This results in increased reactivity, which is observed as enhancement. In contrast, sequestration of cations makes them unavailable for optimum enzyme activity of the LAL cascade, resulting in inhibition. To address problems with testing heparin, more than 20 years ago Associates of Cape Cod, Inc. changed the formulation of the Pyrotell® gel-clot reagent, which is still the gel-clot reagent of choice for this product. Once again, interference is usually overcome by dilution of the sample. Cations can be added to compensate for sequestration by chelators, but this complicates testing and is not recommended except as a last resort.

**Blood, Serum, Plasma and Proteins**

Protein based products or proteins in blood and blood fractions may bind endotoxin and render it unavailable for detection in the LAL test. Alternatively, some proteases degrade the proteins of the enzyme cascade while others (e.g. trypsin) activate it. Dilution may help, particularly when the MVD is large. Heat treatment is commonly used to denature the protein in the sample and allow the heat tolerant endotoxin to be detected. This method is effective in the case of trypsin. A word of caution about heat treatment: some proteins bind endotoxin to a greater degree when heated. A heat treatment regime that is successfully employed for testing plasma and sera in our laboratory is as follows:

1. Dilute the sample by a factor of 2 to prevent coagulation upon heating (in some cases a fourfold dilution is required).
2. Add 1 mL sample to a 10 x 75 mm reaction tube (e.g. Pyrotube®) and cover with Parafilm®.
3. Immerse the bottom half of the tube for 2 minutes in boiling water.
4. Remove and cool the sample prior to testing.

Roth et al.[8] found that fourfold dilution of plasma with 0.15 *M* NaCl followed by a 30 minute heat treatment at 60°C to be the most effective of three treatment approaches tested. A wide range of other treatments of blood, plasma and serum have been described, including use of acids, bases, organic solvents, and surfactants, either alone or in combination[9].

Caution must be exercised when testing protein solutions or blood products. Contaminant endotoxin in the sample does not always behave in the same way as the added endotoxin in the positive product control (PPC, or spike), which used to check for interference. This phenomenon is only detectable by photometric techniques, not the gel-clot method. When performing photometric tests on a series of dilutions of a sample (unspiked and spiked), determine the dilution at which the PPC is appropriately quantified (the non-interfering concentration). Then look at the results for subsequent dilutions to determine whether endotoxin concentrations in the sample decrease proportionally with dilution. If they do not, there is still interference occurring, despite the PPC being within specification. When interference has truly been overcome, endotoxin in the sample will decrease proportionally with dilution (and PPCs will meet the specification).

**Agents that Denature Proteins**

These include alcohols and phenols and dilution may again solve the problem. Alternatively, volatile organic compounds may be evaporated off as water is added and the endotoxin recovered. The solution should not be evaporated to dryness as it may not be possible to fully recover the endotoxin from the surface.

**Extractables**

Extractables from plastics can interfere with LAL tests. We have seen several instances of this. We have reported a water soluble substance extracted from polypropylene tubes that was highly inhibitory[10]. Triethylamine (TEA, 0.05 %) recovered the activity of endotoxin in the sample. As TEA reduces aggregation state of endotoxin and thus increases the activity, it was hypothesized that the inhibitor

caused the endotoxin to aggregate to the extent that its reactivity was greatly reduced. (Note: Because TEA is basic, it was necessary to use Pyrosol buffer to reconstitute the LAL reagent.) If a sample is treated to recover the activity of endotoxin it is important to control for such effects. It may be necessary to dilute endotoxin standards in the same solution if the treatment affects endotoxin activity. This is only practicable in photometric methods as there is no requirement to match label claim sensitivity. It is analogous to the use of product standard curves, which are discussed below.

**Lipids and Liposomes**

Some drug products are dissolved in oils, such as sesame oil, while others are enclosed in liposomes. Perhaps surprisingly, some products dissolved in oils can be diluted in water (LRW) and tested in the normal way, as endotoxin may partition into the aqueous phase during dilution. Verify (and validate) this by spiking the product with endotoxin and demonstrate recovery. In other cases, a surfactant may be used to disperse a lipid, e.g. 0.01% sodium desoxycholate (which is slightly inhibitory). Piluso and Martinez[11] report that sodium dodecyl sulfate (SDS) or CHAPS may be used to disrupt liposomes to facilitate their testing. CHAPS is recommended for liposome formulations containing substantial cholesterol concentrations. Non-ionic detergents such as Triton-X were reported to be unable to disrupt liposomes. These authors caution that polyoxy-ethylene-10-laurel ether (PELE) denatures LAL at low concentrations.

## Other Strategies for Overcoming Interference

Ultrafiltration can be used to separate lower molecular weight interfering substances from endotoxin in the sample. One product, the Sartorius Ultrasart® D-20, in which the membrane has a 20 kD molecular weight cutoff, is designed specifically for this purpose and has been used effectively in our laboratory.

Advantage can be taken of the different formulations of the LAL reagents for different test methods. The reagents often vary in their susceptibility to a particular interference. If interference cannot be overcome by one LAL test method, it is quite possible that it can be addressed with a different method. The differing susceptibilities to interference of three LAL methods is illustrated for two drug products in Table 1.

**Table 1:** Dilutions required to overcome interference for two injectable dug products

| | Penicillin | Clindamycin |
|---|---|---|
| Gel-clot | 16 | 32 |
| Chromogenic | 10 | 20 |
| Turbidimetric | 200 | 100 |

It should be noted that kinetic turbidimetric tests reported in Table 1 were conducted in the LAL-5000® tube reader at a sample/LAL ratio of 4:1. This comparison was made before the advent of the Pyros Kinetix® tube reader, in which a ratio of 1:1 can be used. The higher LAL/sample ratio of 1:1 often reduces the amount of dilution required to overcome interference. In our laboratory we recently found that changing the sample/LAL ratio from 4:1 to 1:1 in the Pyros Kinetix eliminated interference without further dilution and attendant loss of test sensitivity.

It was noted above that use of a more sensitive LAL reagent or test method increases the scope for dilution (i.e. the maximum valid dilution increases), as is illustrated in Table 2.

**Table 2:** Maximum valid dilutions (MVDs) for the three methods

| | Penicillin | Clindamycin |
|---|---|---|
| Gel-clot (Pyrotell, 5 mL) ($\lambda$ = 0.03 EU/mL) | 640 | 2,784 |
| Chromogenic (Pyrochrome) ($\lambda$ = 0.005 EU/mL) | 4,000 | 17,400 |
| Turbidimetric (Pyrotell-T) ($\lambda$ = 0.001 EU/mL) | 20,000 | 87,000 |

($\lambda$ = test sensitivity, i.e. the minimum detectable endotoxin concentration)

For these drugs, interference was overcome by dilution well within the limit of the MVD and both products were validated by all three methods.

If interference cannot be overcome, a product standard curve can be utilized for photometric (but not gel-clot) methods. A series of standard endotoxin concentrations (which will be used to produce the standard curve) are prepared in clean (free of detectable endotoxin) product at the same concentration as that of the sample to be tested. Thus, the degree of inhibition/enhancement is then the same for the standards and for the product. There are two ways to do this.

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Standards can be diluted in the sample to be tested, which requires that the sample contain little background endotoxin in order to give a valid test. The method works well for releasing clean product, but not for quantifying endotoxin concentrations. The alternative approach is to use a reference lot of clean product, which requires that the degree of interference is consistent between lots. The use of product standard curves is addressed in the FDA Guideline on LAL testing. However, it is not generally recommended and should only be used as a method of last resort.

## Validation of Sample Treatment

The three harmonized pharmacopeial endotoxins test chapters require demonstration (i.e. validation) that "treatments, such as filtration, neutralization, dialysis or heating" remove interference but not endotoxin. To do this, the USP BET states, "… perform the assay … using the preparation to be examined to which USP Endotoxin RS has been added and which has been subjected to the selected treatment." Equivalent wording is included in the EP and JP.

## Conclusion

An understanding of the chemistry of the test sample and its possible effects upon endotoxin and/or LAL, can aid in overcoming interference problems. Dilution is always the technique of choice and should be attempted first. A more sensitive gel-clot reagent, or a lower concentration range standard curve for photometric methods, results in a greater MVD and increased scope for dilution. Secondly, use of a reconstitution buffer may assist in overcoming interference, even if pH does not appear to be an issue. Dilution and use of a buffer in accordance with the product insert does not constitute a "treatment" that requires validation other than the normal "Interfering Factors Test."

The degree of interference may be less with a different test method. Also, as the resolution of the gel-clot test is limited by the use of twofold dilutions, interference may be less readily detected by this method. The photometric methods, with their greater sensitivity, allow for greater dilution, which often enables interference to be overcome. Chemical additions or modifications of the sample can usually be avoided, as can product standard curves, but these are approaches that can help with a particularly difficult sample.

Finally, a powerful weapon in the fight against interference is the expertise of Associates of Cape Cod, Inc. Our Technical Service representatives can suggest testing strategies and our Contract Test Service (CTS) can develop test methods and conduct validations, as well as perform routine testing. CTS has a wealth of experience with samples ranging from drug products, medical devices and biologicals to air samples and oils. We are no further than a telephone call or email away.

## References


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# LAL News and Events

**NOVEMBER**

**Society for Glycobiology Annual Meeting**
November 9-11, 2005
Park Plaza Hotel
Boston, MA
*Booth: 1*

**Eastern Analytical Symposium**
November 14-17, 2005
Garden State Convention Center
Somerset, NJ
*Booth: 138*

**DECEMBER**

**American Society for Hematology (ASH) Annual Meeting**
December 10-12, 2005
Georgia World Congress Center
Atlanta, GA
*Booth: 3012*

**Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) Annual Meeting**
December 16-19, 2005
Washington Convention Center
Washington, DC
*Booth: 1038*

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**Customer Service:** custservice@acciusa.com **Technical Service:** techservice@acciusa.com **www.acciusa.com**

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ASSOCIATES OF CAPE COD INCORPORATED

**Corporate Headquarters**
Associates of Cape Cod, Inc.
124 Bernard E. Saint Jean Drive,
East Falmouth, MA 02536
**T** (508) 540–3444
**F** (508) 540–8680

**UK Office**
Associates of Cape Cod Int'l Inc.
Deacon Park, Moorgate Road,
Knowsley, Liverpool L33 7RX
United Kingdom
**T** (44) 151-547-7444
**F** (44) 151-547-7400

**German Office**
Pyroquant Diagnostik GmbH
Opelstrasse 14
D-64546 Mörfelden-Walldorf,
Germany
**T** (49) 61 05-96 10 0
**F** (49) 61 05-96 10 15