# Variability in the LAL Test

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*[EDITOR'S NOTE: This is the third in a series of three presentations from the "LAL Panel Discussion" at the PDA Spring Meeting, New York, NY, March 1989. They are being published as a group in this issue.]*

One of the most difficult problems facing the LAL user is assessing the variability of the test "in our/my hands." I have taken some common sources of variability and divided them into four major categories: *1*) Reagent Variability, *2*) Method Variability, *3*) Product Variability, and *4*) Laboratory Variability. The products, methods, and reagents used as examples below are meant to illustrate a point, and are not meant to define testing trends or endorsements for any particular product, method, reagent, or combination thereof.

## 1. Reagent Variability

The greatest source of reagent variability is the differences in formulation of lysates from different manufacturers. Extraction procedures and final product formulations are proprietary, and differ in many respects including buffering capacity, the presence and concentration of surface active agents, and divalent cation concentration (1). Each of these formulation differences may affect the inhibition/enhancement (interference) profiles observed during the laboratory validation of the product (2-4). Table I summarizes some data for Product X obtained at a recent LAL Users' Group/PDA workshop on product interference with the LAL gel clot test. The MVD for Product X was calculated as 1:200, using a lysate sensitivity of 0.06 EU/mL. The spiked samples each contained endotoxin at a concentration of 2λ/mL. Test inhibition, defined as the inability to detect endotoxin in the spiked samples, was observed on lysates A-C. Inhibition was not observed on Lysate D, however, this lysate exhibited some slight "enhancement," defined for this purpose as a positive response on an unspiked sample. (*Author's note*: Experience in testing of many lots of this natural product from a number of manufacturers suggests that the product may contain low levels of endotoxin. Data obtained above are similar to data obtained prior to the Workshop, suggesting that the "enhancement" was due to a background level of endotoxin rather than contamination of the product or lysate during the test.)

It was known that Product X sequesters calcium. Data summarized in Table II show that the modification of lysates A, B, and C by the addition of calcium dramatically affected the interference profiles of the product/lysate combination.

The interference profiles for Product X are clearly reagent dependent, and suggest that if validation of a product proves difficult, the user might consider trying the validation with reagent obtained from another lysate manufacturer, or using reagent modifiers which may also be used to alter product/lysate compatibility. The user must be cautioned, however, that any reagent modification may alter the lysate sensitivity, so label claim verification studies should be performed on modified reagent.

## 2. Method Variability

A number of LAL testing methods have been proposed over the years, but most laboratories are currently using one of the following LAL methods to test their product: get clot, turbidimetric (including kinetic/turbidimetric), or chromogenic (including kinetic/chromogenic) (5-8). It is generally recognized that lysate/product compatibility can change with the test method chosen by the laboratory, and FDA guidelines for the validation of the LAL test require that products be completely revalidated when the method is changed (9). For example, the gel-clot data obtained for Product X using lysate manufacturer D's reagent showed that a 1:10 dilution was sufficient to overcome test interference (Table I). Preliminary kinetic/turbidimetric data gathered using lysate from the same manufacturer and the same lot of Product X indicated that the non-interfering dilution could range from 1:100-1:10,000 depending on modification of the lysate (by reconstitution with a buffer), and the ratio of sample to lysate used in the test (Table III). These kinetic assays were all performed by the author using a microtiter plate reader ($THERMO_{max_R}$) and LAL test method developed by Molecular Devices, Inc. of Menlo Park, CA. All lysate was licensed for turbidimetric methods. Standard curves ranged from 0.03 EU/mL-1.0 EU/mL, and met all parameters set forth in the FDA Guideline. The MVD for these experiments was 1:400, based on $\lambda = 0.03$ EU/mL.

## 3. Product Variability

In 1984, Chris Twohy and co-workers at FDA published data obtained from testing 333 different parenteral products on the gel-clot test (3). Of these products, 236 or approximately 71% required some dilution or other test modification to overcome interference. Slight lot-to-lot variations in a parenteral product's pH, salt concentration, divalent cation concentration, buffers, preservatives,

Received March 20, 1989. Accepted for publication November 2, 1989.
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TABLE I. Gel Clot Inhibition/Enhancement Screen for Product X

| | | Product X Dilution | | | |
|---|---|---|---|---|---|
| | | Undiluted | 1:10 | 1:100 | 1:1000 |
| **Lysate** | | | | | |
| A | spiked | – | – | + | + |
| | unspiked | – | – | – | – |
| B | spiked | – | – | – | + |
| | unspiked | – | – | – | – |
| C | spiked | – | – | – | – |
| | unspiked | – | – | – | – |
| D | spiked | + | + | + | + |
| | unspiked | + | – | – | – |

MVD = 1:200

TABLE II. Gel Clot Inhibition/Enhancement Screen for Product X Lysate Modified

| | | Product X Dilution | | | |
|---|---|---|---|---|---|
| | | Undiluted | 1:10 | 1:100 | 1:1000 |
| **Lysate** | | | | | |
| A | spiked | – | + | + | + |
| | unspiked | – | – | – | – |
| B | spiked | – | – | + | + |
| | unspiked | – | – | – | – |
| C | spiked | – | – | + | + |
| | unspiked | – | – | – | – |

MVD = 1:200

TABLE III. Gel Clot vs. Kinetic/Turbidimetric Data for Product X

| Method | Non-Interfering Concentration (HIC) |
|---|---|
| Gel Clot | 1:10 |
| Kinetic/turbidimetric 1:1 sample:lysate | $1:10^3$ |
| Kinetic/turbidimetric + buffer 1:1 sample:lysate | $1:10^2$ |
| Kinetic/turbidimetric + buffer 4:1 sample:lysate | $1:10^4$ |

MVD = 1:400

raw material source, and even possible extraneous endotoxin may affect its lysate compatibility profile. For this reason, it is recommended that the testing laboratory consider a number of suggestions to minimize test variation due to product chemistry:

1) Testing at either the determined non-interfering concentration (NIC) or the calculated maximum valid concentration (MVD) may not allow for testing differences due to fluctuation in product chemistry. The choice of a test concentration below the MVD but above the NIC will allow for maximum testing flexibility.
2) Pooling samples and testing at the MVD must be avoided. Any endotoxin contamination in one unit will automatically be diluted by pooling and may not be detected. If samples are pooled, they should be tested at a concentration that will allow for the detection of endotoxin in the "worst case" which is contamination in only one unit at the endotoxin limit.
3) Searching for the best product/lysate or product/method match should be considered, especially in cases where a product has proven difficult to validate. A parenteral product may need dilution up to the MVD for a valid test on one lysate, but may be tested at a much lower dilution on another reagent. Some classes of products may be more amenable to testing by one method than another.
4) The product formulation should be well understood before beginning the validation procedure and followed through process changes to anticipate incompatibility problems.

## 4. Laboratory Variability

The details of the standard operating procedures employed by the laboratory vary from company to company, and may even differ somewhat between laboratories in the same company. Laboratory variability is difficult to assess, and is perhaps the easiest to overlook.

1) It is known that the type of glass and plasticware used by the laboratory may affect the outcome of tests. Dr. James F. Cooper, President, Endosafe, Inc., has presented data (above) suggesting that polypropylene plastic tubes routinely used for the collection and storage of samples or Water for Injection (WFI) may cause interference problems. Similar observations have been made by Novitsky, *et al.* (10), Dr. Michael Dawson, Associates of Cape Cod (personal communication, submitted manuscript) and many Users' Group members (personal communication). To compound the problem, these phenomena are not necessarily supplier-specific, but may result from lot to lot variation, or even "pockets" within a given lot and supplier. It is suggested that samples from new sources of glass and plasticware be evaluated for interference with the LAL test before use in the laboratory.

2) The specifics of performing an LAL assay may contribute to test variability. Two examples immediately come to mind:
   a) Apparent endotoxin recovery from medical devices or endotoxin indicators is affected by many factors. Spiking and drying techniques and extraction procedures such as the elective use of detergents or other endotoxin dispersing agents, and the degree of sonication/vortexing are all variables in the analysis of these articles (10–12). The use of undefined or undetailed procedures will affect results and may add to inconsistencies between laboratories or even between test runs in the same laboratory.
   b) Dilution schemes are potential sources of variation in an endotoxin assay. It is generally recognized large (≥1:100) dilutions are potential sources of error, so many LAL SOPs limit dilutions to ≤1:10. Likewise, the method used to spike product samples may affect interference profiles—Is the product diluted to the test concentration using an endotoxin solution as the diluent? Are the product and endotoxin initially mixed as concentrates and diluted together? Is the total endotoxin spike added in a small volume once the product is diluted? Each procedure might be valid and might work well on any given day, but employing a number of different methods in the laboratory may be a great source of variability over the longer term.

   It is suggested that laboratory Standard Operating Procedures (SOPs) be standardized as much as possible with regard to dilution schemes and testing steps to avoid excessive variability.
3) Equipment calibration procedures and recalibration intervals vary from laboratory to laboratory. The LAL reaction is time and temperature dependent, so the wrong incubation temperature or uneven heating patterns in a water bath or heating block may contribute to test variation. Periodic documented checks on thermometers and other LAL-related testing equipment may help identify and minimize variability.
4) Water is a variable in the LAL test, especially for those laboratories choosing a quantitative method. One must remember that the endotoxin specification for WFI is 0.25 EU/mL. While the endotoxin content of commercially available WFI is usually very low (<0.03 EU/mL), it may add a significant amount of endotoxin to the low end of a very sensitive standard curve (≤0.01 EU/mL).
5) Because of variation in laboratory SOPs, equipment, diluents, glassware, and plasticware, it is prudent for a parenteral manufacturer to confirm data from validations performed off-site before accepting them and using them for in-house testing.

In summary, any one of the variables mentioned above may or may not affect the outcome of a particular LAL test. Total test variability is additive, and may be the result of a number of subtle differences in reagent formulation and use, test method, product specifications, or laboratory procedures. Laboratory analysts, supervisors, and managers must be aware of these variables and should consider them when investigating an unexpected LAL test result.

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