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Lonza

Overcoming Assay Inhibition or Enhancement

Technical Tips

By Scientific Support, U.S.

Introduction

One of the most time-consuming aspects of endotoxin testing using Limulus Amebocyte Lysate (LAL) is pretreating samples to overcome assay inhibition and enhancement. All assays, independent of methodology, are standardized using endotoxin in water. Therefore, unless your sample is water, some components of the solution may interfere with the LAL test such that the recovery of endotoxin is affected. If the product being tested causes the endotoxin recovery to be less than expected, the product is inhibitory to the LAL test. Products that cause higher than expected values are enhancing. Overcoming the inhibition and enhancement properties of a product is required by the Food and Drug Administration (FDA) as part of the validation of the LAL test for use in the final release testing of injectables and medical devices. Proper endotoxin recovery must be proven before LAL can be used to release a product.

The LAL assay is dependent on the proper activation of the cascade of serine proteases that comprises the lysate. Initiation of the cascade involves recognition of endotoxin in the sample by the first enzyme in the system. Once triggered, the other enzymes are activated in a stepwise fashion. In a gel clot assay, the final step of the cascade involves the cleavage of the clotting protein, coagulogen, and its polymerization into a solid gel. If the endotoxin concentration in the test sample is large enough, the gel will be so solid that it

will remain in the bottom of the tube when it is inverted 180 degrees. The last step in a chromogenic assay is the cleavage of a chromogenic substrate and the resulting release of the chromophore pNA. The yellow color of the solution indicates the presence of endotoxin in the sample. Components of the test sample could cause interference with any of the steps in the cascade, therefore affecting the final result.

Although there are some products that will cause LAL assay enhancement, the majority of LAL interference is due to inhibition. Extreme levels of pH will inhibit gel formation. Highly basic solutions, such as NaOH, could cause non-enzymatic chromogenic substrate cleavage, thus causing a solution to seem more contaminated than it actually is. Products that have a high affinity for divalent cations will chelate or sequester them from the lysate formulation. Without the proper concentration of divalent cations, the enzymes in the lysate cannot function properly and so, will not indicate the endotoxin content. In some instances, the endotoxin can be "hidden" from the LAL by components of, or the very nature of, the test solution. If the LAL does not "see" the endotoxin, the gel will not form or the substrate will not be cleaved. In order to overcome LAL interference, dilution in LAL Reagent Water, within the Maximum Valid Dilution (MVD), is recommended.

Calculating the MVD

$$\textbf{MVD} = \frac{\textbf{ERL}}{\textbf{Lambda}}$$

MVD = Maximum Valid Dilution

ERL = Endotoxin Release Limit (EU/mL)

Lambda = LAL assay sensitivity

- **Gel Clot** = lysate sensitivity
- **All others** = lowest endotoxin standard

Example: 5% Dextrose Injection, ERL = 0.5 EU/mL

Gel Clot Assay: Lambda = 0.06 EU/mL

$$\textbf{MVD} = \frac{\textbf{0.5 EU/mL}}{\textbf{0.06 EU/mL}} = \textbf{8.3}$$

Kinetic-QCL® Assay: Lambda = 0.005 EU/mL

$$\textbf{MVD} = \frac{\textbf{0.5 EU/mL}}{\textbf{0.005 EU/mL}} = \textbf{100}$$

For some products, dilution to the MVD with LAL Reagent Water alone will not be successful in removing all of the LAL interference. In some instances, an alternative diluent is necessary. For example, products that are highly acidic or highly basic may require pH adjustment or dilution with a buffer. Of course, whenever anything is added to a product, steps must be taken to help ensure that no endotoxin is added. Diluents must be made with LAL Reagent Water (or equivalent) in endotoxin-free containers. The endotoxin content of the alternative diluent must be determined prior to use. If endotoxin is detected in the diluent, the endotoxin must be removed. Filtration through a pyrogen-free 10,000-20,000 molecular weight cutoff ultrafilter will remove endotoxin from solutions. Once the solution is free of detectable endotoxin, it can be used to dilute the inhibitory product.

To save customers the time and effort of making endotoxin-free solutions for use as alternative diluents, Lonza offers three solutions for overcoming LAL testing inhibitions. All three of these products are tested for functionality and endotoxin content. Two of these products are simple solutions that will make your LAL testing easier. Catalog No. S50-641 is a 10 mM $MgCl_2$ Solution and Catalog No. S50-642 is a 50 mM Tris Buffer Solution. The other member of the trio, PYROSPERSE®, Catalog No. N188, is our proprietary dispersing agent. The remainder of this article will provide examples of the use of these three products.

Use $MgCl_2$ to overcome chelation effect

Heparin is an anticoagulant. Because LAL is composed of the coagulation system of the horseshoe crab, heparin manufacturers find that their product also inhibits LAL gel formation. Heparin seems to chelate the divalent cations in the LAL formulation, thus depleting the enzymes in the lysate of the cations they need to function properly. Diluting the heparin sample in water diminishes the inhibition, however, the dilution factor cannot exceed the MVD. Reconstituting and/or diluting the heparin in a solution containing divalent cations, such as $MgCl_2$, will help overcome the inhibitory nature of heparin and decrease the necessary dilution factor. The heparin will chelate cations from the diluent. Therefore, when it is mixed with the LAL, the heparin will not remove cations from the lysate. For example, tests showed that when 2 mL of LAL Reagent Water was added to blood collection tubes containing 30 USP units of heparin, the Positive Product Control (PPC) recovery for a tested aliquot was 42%, just below the acceptable range of 50–200% for a Kinetic-QCL® Assay (see Table 1). The heparin is inhibitory to the assay. If 2 mL of 10 mM $MgCl_2$ was added to the heparinized tubes, then the PPC recovery, 118%, is within the acceptable range. Using 10 mM $MgCl_2$ to dilute heparin or other cation-chelating products will help your product easily pass the LAL inhibition/enhancement test.

Adjust the pH with Tris Buffer

The LAL assay functions best in the 6.0 to 8.0 pH range. If a product is outside that pH range, pH adjustment may be necessary. Lonza LAL is buffered, so simple dilution of products in LAL Reagent Water may be sufficient to render the product testable. Adjusting the pH of an unbuffered solution with endotoxin-free acid or base is not recommended. Often times, the desired pH is overshot several times during the adjustment, thus altering the ionic strength of the solution just enough to inhibit LAL functionality. Instead, a buffer in the correct pH range is recommended as a diluent for acidic and basic product solutions. Lonza Tris Buffer, for use with LAL, is a 50 mM solution with an appropriately neutral pH. This Tris Buffer is recommended for use with products that are outside the acceptable pH range. Whereas a 1:10 dilution of such a product in water may remain inhibitory, the same dilution in the Tris Buffer may yield acceptable spike recovery results. Table 1 includes example data. Here, 0.01 N HCl was diluted 1:10 with LAL Reagent Water and tested with the Kinetic-QCL® Assay. The percent recovery of the 0.5 EU/mL PPC was 28%, indicating that the solution is inhibitory to the assay. When the 0.01 N HCl solution was diluted 1:10 with the 50 mM Tris Buffer, the percent recovery was 98%. This is well within the FDA required 50–200% recovery range.

Sample	Diluent	Endotoxin Content	PPC% Recovery
Heparin (30 units)	LAL Reagent Water	<0.005 EU/mL	42%
	10 mM $MgCl_2$	<0.005 EU/mL	118%
HCl (0.01 N)	LAL Reagent Water	<0.005 EU/mL	28%
	50 mM Tris Buffer	<0.005 EU/mL	98%

Table 1.
Examples of the PPC recoveries with LAL Reagent Water and alternative sample diluents. The LAL assay used was the Kinetic-QCL® Assay from Lonza.

Try PYROSPERSE® to solve aggregation problems

Endotoxin is a molecule with a split personality. The polysaccharide portion of endotoxin likes to be in an aqueous environment. The lipid portion, however, is hydrophobic. As a result of its nature, endotoxin monomers will aggregate such that the lipid portion is hidden from the aqueous solution. It is the lipid portion of the endotoxin molecule that activates the LAL reagent. Some products will actually attract and hide endotoxin. In order to detect endotoxin in these products, the endotoxin must be coaxed from its preferred environment. Detergents or surfactants can be used to dissociate endotoxin from itself and products; much like dishwashing liquid disperses grease in a water-filled frying pan. PYROSPERSE®, Catalog No. N188, is a metallo-modified polyanionic dispersing agent. For the gel clot method, a 2% PYROSPERSE® solution is recommended for use as a diluent. A 0.5% solution should be used with the chromogenic assays QCL-1000® or Kinetic-QCL®. Higher concentrations of PYROSPERSE® may inhibit the LAL test. Dilution of a sample with the appropriate concentration of PYROSPERSE® plus a vigorous vortexing can help to dissociate endotoxin aggregates. PYROSPERSE® will change the environment of the solution just enough that the endotoxin is now more "comfortable" and it will expose its lipid portion to the LAL. In 1982, Guilfoyle and Munson published a report documenting that the use of PYROSPERSE® would increase the amount of detectable endotoxin in inhibitory products (Dennis E. Guilfoyle and Terry Munson, "Procedures for Improving Detection of Endotoxin in Products Found Incompatible for Direct Analysis with Limulus Amebocyte Lysate," in: Endotoxins and Their Detection with the Limulus Amebocyte Lysate Test, Alan R. Liss, Inc., New York, 1982). The data included in the report showed that dilution of the inhibitory products in 2% PYROSPERSE® (for the gel clot assay performed) yielded 2 to 175-fold endotoxin detection improvements.

An example of a product that can be successfully LAL tested with PYROSPERSE® is hyaluronic acid. This product will not go into solution in water, even with vigorous vortexing. Hyaluronic acid, 500 mg, was made soluble, with 4% PYROSPERSE® in water. This solution was further diluted to 0.5 ng/mL in water and tested with the Kinetic-QCL® Assay. The percent recovery of the 0.5 EU/mL PPC for this sample was 139%, which is within the acceptable recovery range. Although the PYROSPERSE® concentration used to dissolve the product was higher than what is recommended in the PYROSPERSE® Product Insert, dilution of the hyaluronic/PYROSPERSE® mixture within the MVD with LAL Reagent Water yielded acceptable endotoxin spike recoveries in a Kinetic-QCL® inhibition/enhancement assay.

Final recommendations

It is not appropriate to use the pretreatment on the endotoxin standards. The "gold standard" in LAL testing is endotoxin in water, which is also used for the standard curves required in LAL testing. Pretreatment of samples is necessary to make the samples behave more like endotoxin in water. If the pretreatment is used to prepare the endotoxin standards, the activity of the standards may be altered. This changes the "gold standard" and could adversely affect the assay results.

Inhibition and enhancement testing is an integral part of the validation process for the use of LAL in the final release of products. The United States Pharmacopeia (USP) Chapter 85, Bacterial Endotoxins Test, allows for pretreatment of products to render them testable. As long as it can be demonstrated that the pretreatment allows for the recovery of added (spiked) endotoxin, within the acceptable limits of the LAL method being used, the pretreatment will be acceptable. Once a pretreatment is found that will successfully overcome the inhibition or enhancement of at least three lots of the product, the product can be validated for release with LAL. For routine testing of products, the product must be prepared in the manner in which it was treated to pass the inhibition/enhancement test. Otherwise, a negative result may be mistaken as indicating a lack of endotoxin in the product, when in reality, the negative is a result of inhibition.

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