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**Summary**

A critical part of bacterial endotoxin testing is the choice of accessories that enables data collection free of artifacts and sources of interference. The selection of non-interfering accessories is not only a pharmacopeial directive, but also a regulatory expectation.


MICROBIAL SOLUTIONS

# Limulus Amebocyte Lysate (LAL) Accessories for Bacterial Endotoxin Testing

The generation of valid bacterial endotoxins test (BET) results necessitates robust test procedures, well-maintained equipment, and properly trained personnel. The most expensive LAL test is the one that must be repeated because of invalidity. Accessories play a major role in the quality of results generated in the BET lab. But all too often, cost, not quality, is the driving force in the BET accessory decision-making process.

The root causes of accessory interference may be traced to influences on LAL reagent quality or control standard endotoxin (CSE) potency. Activation of LAL reagent may arise from touch contamination by operators and contact with accessories that contain trace amounts of endotoxin or glucans. Subtle effects may be seen, such as increased background noise in kinetic BET studies or reduced recovery of positive controls. Of course, there can be obvious manifestations, such as gels in the quarter lambda and/or negative controls for gel-clot assays.

CSE fragility, due to the low concentrations inherent in standard solution, can be a risk of artifacts and interference for endotoxin testing. That makes endotoxin susceptible to sources of soluble impurities; notably, extractables from plastics. With time, endotoxin standards seem to disappear due to poorly understood aggregation phenomena. Good accessories and diligent vortex mixing are needed to maintain standard potency. The most common problems are loss of CSE potency or contamination in low-concentration standards for kinetic studies. Since the standard curve is an inverse relationship with reaction time and endotoxin concentration, a weak standard series may result in over-reporting of analysis results and enhancement of the positive controls. More importantly, an over-strength standard series may result in underreporting of analysis results and inhibition of the positive controls.

It is important to interject at this point that the instability in potency described above is a property only of purified endotoxins, such as the lipopolysaccharide (LPS) in CSE reagents (usually derived from *E. coli*). In contrast, environmental (naturally occurring) endotoxin is remarkably stable and dispersible in aqueous solutions. Purification of endotoxin removes proteinaceous components that render LPS poorly dispersible and less stable in potency than endotoxin and leads to increased molecular aggregation, loss of LAL activity and lower toxicity in mammals. If only working with environmental endotoxin, not LPS, no vortex mixer would be needed in the BET lab, because it often retains its potency in simple solutions for years.

## Plastic Supplies

Although the fabrication process of plastic materials should render these items at very low risk, if any, for endotoxin contamination, they may be the most problematic accessories in the BET lab. The harmonized BET chapters warns: "If employing plastic apparatus such as microplates and pipette tips for automatic pipettors, use only that which has been shown to be free of detectable endotoxin and not to interfere with the test."

## Pipettes

Disposable plastic pipettes are notorious for contributing contaminants that at least partially activate LAL reagent. For example, the use of these items for LAL rehydration, sample dilution, and CSE solutions often causes low-level contamination in kinetic LAL studies. Depyrogenated glass pipettes are the best choice for LAL dispensing.

Cotton and other cellulosic materials are particularly problematic for LAL applications. Cotton can contain endotoxin and glucan and is the purest form of cellulose containing substantial amounts of LAL-reactive glucans (LRG). Therefore, pipettes that contain protective cotton or cellulosic plugs should be banned from the BET lab.

## Pipette Tips

Polypropylene pipette tips are universally used to transfer LAL reagent and other test components for LAL testing. To Charles River's knowledge, there is no recent report of interference or contamination assignable to polypropylene pipette tips used only for LAL dispensing. Sterile pipette tips should never be used to rehydrate LAL, and should be avoided for the rehydration of CSE and dilution preparation. A review of historical data regarding lambda and negative controls in BET applications is valuable evidence that a brand of pipette tips has, on the whole, been non-interfering. Never use filter tips, as these have been seen to cause issues.

## Polystyrene Tubes

Another general exception to problematic plastics is sterile, disposable polystyrene tubes, which are universally accepted as a suitable, inert container for preparation of endotoxin standards.

The stability of endotoxin standards in polystyrene tubes is equivalent to borosilicate tubes, in our hands. Container-related loss of CSE potency was initially attributed to adsorption by polypropylene. Later studies by others, including our lab, found that the loss was actually caused by unknown powerful inhibitors that were extracted from the polypropylene containers. The choice of non-glass containers for collection of water samples requires validation to assure the absence of inhibitors. The ideal collection vessel would be non-breakable, heat-stable, and free of BET-interfering extractable agents. Part of validation of a water system is the identification and validation of specific noninterfering containers for collection and storage of water samples.

## Multi-Pipetting Syringes

A common practice in the BET lab is to use a sterile multi-dispenser to dispense LAL reagent, such as the Eppendorf® biopore 5 mL Combitip®. In Charles River's experience, these devices are endotoxin-free, as indicated by consistently obtaining non-reactive negative controls in gel-clot and kinetic BET studies. There have been reports of LAL contamination problems with similar devices made by other suppliers. Charles River's experience also shows that the use of a 0.5 mL Combitip® for inoculating 10 $\mu$L endotoxin spikes is the most accurate, efficient, and robust method for preparing hot-spike positive controls in gel and kinetic BET methods.

## Microplates

Polystyrene microplates may be the item of most interest for quality control because more than one half of all LAL tests are conducted in a microplate. A clean environment is needed for microplate fabrication to avoid dust and dirt that convey endotoxin and LAL-reactive material. Some brands of microplates are troubled by the occurrence of "hot wells," where there seems to be random occurrence of overreactive wells. A suitable microplate for LAL work will not yield nonspecific gels or hyper-reactivity in samples or standards during incubation. Therefore, a screening procedure for a microplate supplier should assure that microplate reactivity is less than lambda, the lowest concentration in a kinetic BET series.

The screening procedure used by Charles River to release sterile microplates and certify nonreactivity is the following:

1. Randomly add lambda to 4 wells.

2. Add LRW to the remaining wells.

3. Add LAL reagent to all wells.

A microplate meets the acceptance criteria if the onset times for 99% of the wells containing LRW fail to react at a time less than the wells containing lambda concentrations.

## Summary

Certain plastic accessories cause contamination of LAL reagent and release inhibitors that modify the dispersion of CSE in working standards. Polypropylene pipette tips, polystyrene tubes, and polystyrene microplates that have noninterfering properties are available. Glass pipettes are the best option for rehydration of LAL reagent. While borosilicate glass is the gold standard, it still needs to be free of interfering factors.

It is important to know how sterile plasticware has been tested and to what limit, beyond the manufacturer's claims. In addition, incoming material control is important; issue-free use of an item or brand of items for years does not guarantee trouble-free testing for time immemorial, as plastics change.

# LAL Reagents and Accessories

| Product Line | Product | | Code |
|---|---|---|---|
| Depyrogenated Glass Pipettes | 1 mL, in foil | Pack of 10 | P100 |
| | | Box of 50 | P100 |
| | 2 mL, in foil | Pack of 10 | P200 |
| | | Box of 50 | P200 |
| | 5 mL, in foil | Pack of 10 | P500 |
| | | Box of 50 | P500 |
| | 10 mL, in foil | Pack of 10 | P1000 |
| | | Box of 50 | P1000 |
| Eppendorf® Pipette Tips | 2-200 µL* | Pack of 50 | D100EA |
| | 2-200 µL | Pack of 100 | D200 |
| | 2-200 µL* | Case of 1000 | D1000 |
| 96-Well Endosafe® Plates** | 96-well polystyrene plate (certified to 0.005 EU/mL) | Each | M9005 |
| | | Case of 50 | M9005 |

* Certification of Quality not included.

** Tissue culture treated.

| Product Line | Product | | Code |
|---|---|---|---|
| Control Standard Endotoxin (CSE) *E. coli* | CSE – 500 ng per vial** | Pack of 6 | E110 |
| | | Box of 100 | E110 |
| | CSE – 10 ng per vial** | Pack of 6 | E120 |
| | | Box of 100 | E120 |
| | Positive control (for single test) | Pack of 25 | PC100 |
| Reference Standard Endotoxin (RSE) | RSE USP – 10,000 EU per vial | | E150 |
| Extended CSE (CXE) Dilution Kit** | CXE Dilution Kit<br>1 × 10 ng CSE<br>1 × 60 mL stabilizing solution<br>1 pack of 11 of 16 × 100 mm capped tubes | | E140 |
| LAL Reagent Water*** | 30 mL bottle (<0.001 EU/mL) | Case of 12 | W130 |
| | 50 mL bottle (<0.001 EU/mL) | Case of 12 | W120 |
| | 100 mL bottle (<0.001 EU/mL) | Case of 12 | W110 |
| | 500 mL bottle (<0.001 EU/mL) | Pack of 6 | W150 |

** Lot-specific Certificate of Analysis included when reagents are purchased together.

*** Certification of Quality included.

| Product Line | Product | | Code |
|---|---|---|---|
| LAL Buffers* | 5 mL 0.25 M Tris buffer | Pack of 6 | BT101 |
| | 30 mL 0.1 M Tris buffer | Case of 12 | BT103 |
| | 5.5 mL 0.1 M Tris buffer | Pack of 6 | BT105 |
| | 30 mL 0.05 M Tris buffer | Case of 12 | BT106 |
| | 4 mL 0.5 M $MgSO_4$, 1 M Tris buffer | Pack of 6 | BC1000 |
| | 5.2 mL endotoxin-specific buffer | Pack of 6 | BG120 |
| | 30 mL bio-dispersing agent | Case of 12 | BD100 |
| Endotoxin Indicators** | 10,000 EU | Case of 12 | EVV10K |
| | | Box of 100 | EVV10K |
| | 100,000 EU | Case of 12 | EVV100K |
| | | Box of 100 | EVV100K |
| | 1 Million EU | Each | EVV1M |
| | 2.5 Million EU | Each | EVV2.5M |
| | 10 Million EU | Each | EVV10M |
| Tubes | 10 × 75 mm capped flint glass tubes, boxed | | T100 |
| | 10 × 75 mm flint glass tubes, in foil | | T200 |
| | 13 × 100 mm borosilicate glass tubes, in foil | | T300 |
| | 10 × 75 mm borosilicate glass tubes, in foil | | T400 |
| | 8 × 75 mm borosilicate glass tubes | | T500 |
| | 18 × 150 mm borosilicate glass tubes, in foil | | T600 |
| | 16 × 125 mm pack of 12 | | T900 |
| | 16 × 90 mm screw-cap borosilicate glass tubes, boxed | | TL700 |
| | 12 × 75 mm borosilicate glass tubes, in foil | | TL1000 |
| | 10 × 75 mm screw-cap borosilicate glass tubes, boxed | | TL1200 |

* Please contact Technical Support before using buffers. Additional buffers are available for specific testing needs.

** For dry heat oven validations

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