Endotoxin Testing Best Practices & Troubleshooting Guide: Sievers Eclipse Bacterial Endotoxin Testing (BET) Platform

This guide was designed to help users with BET, covering essential best practices and practical guidance across a wide array of general endotoxin testing topics.

Visual Inspection of Microplates:

- Do not attempt to use previously run plates.
- Set up the assay and start the test within the software, following all on screen instructions.
- Prior to opening, verify that plate packaging is sealed and free from damage.
- Prior to running an assay, carefully inspect the plate for visible defects such as scratches or smudges over the optical wells. Be sure to insert injection ports facing up.

Inspection procedures in the event of a deviation/invalid:

1) Use a magnifier to inspect the optical reaction wells for proper liquid volumes if an invalid (i.e. split well) occurs. This could be an indication of improper pipetting. See Figure 1 Below
2) If a fluidic failure occurs, examine optical wells of the plate to determine if there is liquid present
    a) If liquid proportions are not equal, this could be contributed to either not enough sample/lysate volume reaching the well or sample interference
3) During plate registration, if the Eclipse analyzer rejects a plate it could be due to a few reasons:
    a) The plate has been used and the optical wells have liquid in them
    b) There is a scratch or smudge over an optical well or wells that would interfere with the reading. Visually inspect all optical wells to determine if one of these reasons are present.

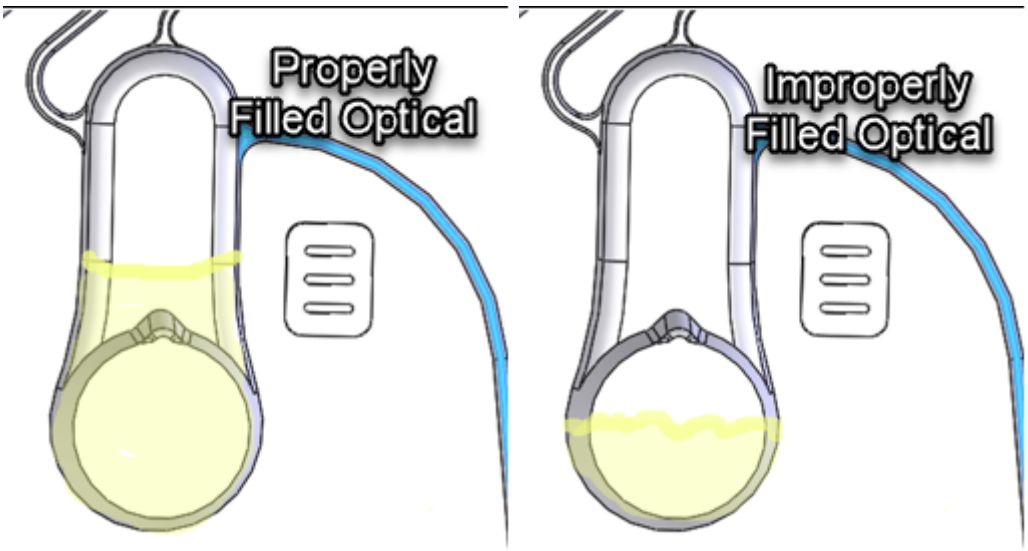

Figure 1 - Optical Well liquid volumes

LAL Reagent Handling:

Rehydration Protocol:

- We **highly recommend** rehydrating LAL using borosilicate, depyrogenated, serological glass pipettes to minimize particulate introduction and potential plastic interference.
- After rehydration, **discard** the stopper and cover the LAL with Parafilm to **avoid** LAL activation issues
- Immediately place any unused LAL in the refrigerator or aliquot it out into an appropriate container to freeze.
    a. Note: do not freeze and thaw LAL more than one time.

Manufacturer Compatibility:

- Use LAL and LAL Reagent Water (LRW) from the same manufacturer. Each manufacturer formulates their LAL differently with specific requirements for container type, pH, and buffering capacity.
- Note that different LAL formulations may have varying sensitivity to interferences.

Sample Preparation:

Dilution Best Practices:

- Use larger volumes for dilutions to increase robustness and reduce potential error.
- *Example:* For a 1:10 dilution, add 1 mL of sample to 9 mL of LRW rather than using smaller volumes.

Pre-Testing Requirements:

- Vortex each sample for 30-60 seconds prior to adding it to the microplate. This ensures homogeneous dispersion of endotoxin, which naturally aggregates.
- Practice strict aseptic technique when preparing samples, making dilutions, and collecting samples.
- It is recommended to open and use LRW in the same shift. If this is not possible, it is recommended not to use LRW that has been open longer than 24 hours.

Sievers Eclipse Platform-Specific Best Practices

Understanding the Platform

The Sievers Eclipse was designed to minimize errors and prevent contamination**, but since it uses LAL it is still subject to variability and interferences of the kinetic chromogenic cascade**. Following proper techniques and using recommended accessories is critical for achieving repeatable, robust, and precise results**.**

Recommended Accessories

Pipettes and Tips:

- Use Eppendorf Biopur 200 µL and 1000 µL pipette tips with Eppendorf Biopur pipettors.
- The Eclipse microplate wells are specifically shaped to fit perfectly with Biopur pipette tips, creating a proper seal for accurate sample dispensing.
- Using other pipette tips may not create a perfect seal, which can impact dispensing accuracy and the vacuum created during sample addition.

Free of Detectable Endotoxin:

- Ensure all accessories (pipette tips, dilution tubes, containers) are **free of detectable endotoxin down to the sensitivity level to which you are testing.**
- *Example:* If running a standard curve to 0.005 EU/mL, pipette tips must be rated to < 0.005 EU/mL using LAL.

Validation Studies:

- Perform spiking studies before using any accessory to ensure no endotoxin adsorption or interference occurs.
- Conduct hold-time studies if samples will be held for a period before testing.

Proper Pipetting Technique

Key Differences from 96-Well Plates:

- The Eclipse microplate is designed for the pipette tip to **be gently inserted into the well** (unlike traditional 96-well plates).
- Pipette straight up and down, not at a 45-degree angle.
- This vertical approach minimizes ergonomic injuries, though it may require a technique adjustment for experienced analysts.

Microplate Handling:

- When removing the microplate from packaging, avoid touching the bottom of the plate or sample wells.
- Fingerprints on optical wells can interfere with readings.
- Touching sample wells can introduce contamination.

Understanding Interferences-Common Sources of Interference

Most samples interfere with LAL's enzymatic reaction, manifesting as either inhibition or enhancement.

Interference Type	Definition
Inhibition	**Positive Product Control (PPC) Recovery <50%, indicating that a substance within the product is interfering with (inhibiting) the Limulus Amebocyte Lysate (LAL) enzymatic reaction**
Enhancement	Positive Product Control (PPC) Recovery >200%, indicating that a substance within the product is interfering with (enhancing) the Limulus Amebocyte Lysate (LAL) enzymatic reaction.

Material-Related Interferences:

- **Polypropylene plastic:** Can adsorb endotoxin or release particulates (avoid use **of polypropylene serological pipettes, dilution tubes, sample containers)**
- **Polystyrene plastic:** Acceptable for BET applications.
- **Glass:** Use only depyrogenated, borosilicate glass when needed; other glass types can cause **in**terference.
- **Metals:** Can interfere with the assay.

For comprehensive information, reference USP <1085> or the Sievers Eclipse Inhibition/Enhancement **A**pplication Note.-----Troubleshooting Invalid Results Common Causes of Invalid Assays

Investigating an Invalid Result

Definitions and assay criteria

- PPC percentage outside the 50-200% range.
- Sample CV% or PPC CV% outside the user-defined range.
- Standard curve CV% outside acceptable limits.
- Negative controls reacting before the lowest standard.
- Out-of-specification results **- sample** exceed**s** the endotoxin limit.
- Hot wells/split wells **-**one sample well reacts while the other does not.

Immediate Actions

1. **Visual inspection:**

- Visually inspect the Eclipse Microplate for bubbles, particulates, uneven liquid levels using a magnifying glass or microscope. This should be performed immediately after the assay completes, as there will be settling of the liquid.
- Pay close attention to the segment in which there was an invalid, if needed take pictures of the optical wells and their fill levels.
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2. **Document:**

- All materials used with the assay:
    - Pipette tips: Lot number, Expiration date, Date Opened
    - LAL Reagent Water: Lot number, Expiration date, Date Opened
    - Dilution tubes: Lot number, Expiration date, Date Opened
    - Pipettor: Calibration date
    - Buffers: Lot number, Expiration date, Date Opened
    - Sampling Containers: Lot number, Expiration date, Date Opened
    - Any other accessories that came into contact with the sample or materials
- Environmental Conditions
- Analyst training information
- Any observed anomalies

3. Preserve:
    - Remaining sample- Store at 2-8°C while investigation is ongoing. It may be needed to perform a retest with the original sample
    - Eclipse Microplate- store during investigation
    - LAL and LAL Reagent Water- cover LAL in parafilm and store at 2-8°C. Quarantine the LAL Reagent Water during investigation, it may be necessary to test the LAL Reagent Water as a sample to determine whether contamination is present or not.
    - Pipette tips and other accessories- quarantine as part of the investigation. Accessories may require testing to determine whether there is contamination.

Root Cause Investigation

Troubleshooting Split Wells/Hot Wells

Hot wells (also called **split** wells) occur when one sample well reacts while the duplicate does not, typically resulting in an invalid sample CV%.

Root Causes and Solutions:

Cause	Solution
Low-level contamination from accessories	Validate all pipette tips, dilution tubes, and sample containers through spiking studies.
Contaminated LRW	Use fresh, validated LRW; store properly to prevent contamination.
Physical contamination	Avoid touching pipette tips or sample wells; maintain aseptic technique.
Beta-glucan from filters	Use glucan blocking buffer from your LAL manufacturer.
Insufficient volumes	Ensure adequate LAL and sample volumes are dispensed.
Particulates	Use appropriate pipettes and ensure samples are free of particulates.
Manufacturing contamination	Use validated, certified depyrogenated accessories.
Premature assay termination	Allow sufficient time for both sample wells to react.
Sample interference	Dilute samples appropriately; consider pH or other buffers if needed.
Endotoxin aggregation	Vortex samples for 30-60 seconds before testing.

Troubleshooting Invalid PPCs Understanding PPC Failures:

- **Most samples interfere with LAL, which is why dilution is often required. Even water systems can show interference, demonstrated by PPC percentages outside the acceptable range.**

Enhancement (>200% PPC):

- **Cause:** Beta-glucans enhance the assay.
- **Solution:** Use glucan blocking buffer from your LAL manufacturer (do not use buffers from other manufacturers).

Inhibition (<50% PPC):

- **Cause:** Inhibitory sample components.
- **Solutions:**
    - Increase sample dilution.
    - Use pH buffer or other appropriate buffers.
    - Perform inhibition/enhancement studies to determine optimal dilution.

Troubleshooting Invalid Sample Results seen on Eclipse Reports:

Note: this is not a comprehensive list of causes and solutions, this list contains the most common reasons.

Cause	Cause / Solution
Invalid CV%	**Under pipetting, inadvertently contaminating the sample or pipette tip, sample interference, endotoxin aggregation** **Solution: Ensure pipette is set to 55uL; use validated, uncontaminated LRW; maintain aseptic technique; proper sample preparation and screening; proper injection technique (straight up and down)**
Invalid PPC%	**Sample interference showing inhibition or enhancement (<50% or >200 %).** **Solution: Dilution in LRW, Glucan buffer, pH adjustment buffers**
Fluidic Failure	**Not enough sample or LAL volume injected into the sample or LAL port / missed sample segment**
Release Limit Exceeded	**The endotoxin limit for a sample is exceeded or above the defined alert/action limit** **Solution: Internal investigation to determine root cause of sample contamination**

Troubleshooting Invalid Standard Curves on Eclipse: Common Causes

Cause	Solution
Contamination	Inadvertent contamination of LRW used for rehydrating the standard curve. **Solution:** Use validated, uncontaminated LRW; maintain aseptic technique.

Volume Errors	Insufficient volumes of LRW during standard preparation or accidentally pulling LRW back into the pipette tip. **Solution:** Carefully control pipetting technique; verify volumes.
Insufficient LAL	Not dispensing adequate LAL volume. **Solution:** Verify LAL volume dispensed; check pipette calibration.

Corrective Actions

- **Pipetting Technique:** Retrain analyst- ensure pipettor is not at 45 degree angle, pipette tip is placed in the sample well, ensure the analyst is pipetting down to the second stop.
- **Sample Particulates:** Filtration may be required if the sample has a lot of particulates. Also, inspect plastic containers to ensure they are not emitting plastic particles.
- **Matrix interference:** Perform further dilutions in LAL Reagent Water or investigate whether other sample preparation techniques are required for this sample. Obtain a fresh sample to determine if there was some contamination in the original sample.
- **Reagent issues:** Prepare a fresh vial of LAL.
- **Instrument (unlikely):** Run OVP/TVP; 1:1 Ratio; contact Sievers Support
- **Unknown cause:** Retest with all fresh materials (accessories, sample, LAL, LRW); potentially retest with new analyst

Quick Reference Checklist:

Before Starting Your Assay:

- ☐ LAL and LRW are from the same manufacturer.
- ☐ All accessories are rated for your target endotoxin level.
- ☐ Accessories have been validated through spiking studies.
- ☐ Using Eppendorf Biopur pipettes and tips with the Eclipse platform.
- ☐ LAL rehydrated with borosilicate, depyrogenated glass pipettes.
- ☐ LAL covered with Parafilm after stopper removal.

During Sample Preparation:

- ☐ Using larger volumes for dilutions.
- ☐ Maintaining aseptic technique.
- ☐ Vortexing samples for 30-60 seconds before testing.
- ☐ Not touching microplate wells or the bottom of the plate.
- ☐ Pipetting straight up and down (not at a 45-degree angle).

If You Encounter Problems:

- **Split Wells:** Check for contamination sources, verify volumes, ensure adequate vortexing, allow sufficient reaction time.
- **Invalid PPCs:** Increase dilution, consider beta-glucan interference, evaluate the need for buffers.

Invalid Standard Curve: Verify LRW quality, check rehydration technique, confirm adequate volumes.

Additional Resources:

For more detailed information, please consult:

- USP <1085> for interference guidance
- Sievers Eclipse Inhibition/Enhancement Application Note
- Your LAL manufacturer's technical documentation
- Contact your Sievers technical support team for platform-specific questions

-----This guide is intended for trained laboratory personnel performing bacterial endotoxin testing using the Sievers Eclipse BET Platform. Always follow your laboratory's standard operating procedures and applicable regulatory ***requirements.***

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