TECHNICAL TIP

BACTERIAL ENDOTOXIN TESTING

Pyrogens are substances that can induce a fever response in the body, and sources can be either microbial or non-microbial. The focus of this TechTip is the testing associated with a microbial pyrogen, bacterial endotoxin.

Bacterial endotoxins are membrane bound lipopolysaccharides in the cell wall of Gram-negative bacteria. The toxicity of these lipopolysaccharides is attributed to the Lipid A component of the lipopolysaccharide.

The Bacterial Endotoxin Test is used to measure pyrogenic (fever-inducing) substances derived from Gram-negative bacterial contamination. Within the context of medical device and pharmaceutical manufacturing, Gram-negative bacterial contamination is generally isolated from water sources. If certain levels of these chemicals are present on or in the medical device or pharmaceutical, they can lead to significant patient harm or death depending on the type of patient contact. It is critical that devices with contact to the circulatory system, cerebrospinal fluid, the eyes, implants, and other parts of the body that may be susceptible to endotoxin be monitored regularly to ensure patient safety.

Details regarding endotoxin testing are found in Pharmacopeias (USP<85>, USP<161>, USP<1085>, EP 2.6.14, etc.).

Limits are described in ANSI/AAMI ST72, *Bacterial Endotoxins – Test Methods, Routine Monitoring, and Alternatives to Batch Testing,* for the following types of devices:

Device Type	Limit (Endotoxin Units/device)
Circulatory Contacting	< 20 EU/device
Cerebrospinal Fluid Contacting	< 2.15 EU/device
Intraocular Devices	< 0.2 EU/device

Samples are described in ANSI/AAMI as:

- < 30 batches: *2 samples*
- 30-100 batches: *3 samples*
- ≥ 101 batches: *3% of batch up to a maximum of 10 samples*

Samples are defined in USP<85>, USP<161>, USP<1085> as:

- 3-10 samples

Additional alert and action levels may be established by the manufacturer to monitor and trend, assuring control of the manufacturing process.

Bacterial endotoxins are heat stable chemicals and may exist after the death or sterilization of the bacterial source. In living bacteria, endotoxin can be continuously released by the cell. Given the size of the endotoxin, filtration is generally not an effective method of removal. Strong acids, strong bases or high temperatures may be required to reduce endotoxin contamination on a product. These methods, however, can have adverse effects on common medical device component materials.

The reagent used in the Bacterial Endotoxin Test, Tachypleus or Limulus Amebocyte Lysate (TAL/LAL), is isolated from the hemolymph of either the Southeast Asian (Tachypleus) or Atlantic/American (Limulus) genus of horseshoe crab.

Utilizing the clotting factor of the horseshoe crab, the Bacterial Endotoxin Test utilizes the combination of a liquid or liquid extract of a product/device combined with the TAL/LAL reagent to detect or quantify bacterial endotoxin in the sample through multiple methods.

Extractions for endotoxin are typically performed on the patient-contacting portions of the device (direct or indirect contact) or those labeled as non-pyrogenic. Extractions are typically performed for either 15 minutes at 37°C or one hour with 37°C water extracted at room temperature. Intraocular products may require one hour extraction at 37°C with agitation due to endotoxin sensitivity in the eyes. Testing of process water does not require extraction. Some materials may inhibit or enhance the result due to the presence of beta glucans. Beta glucan interference may require special buffers to ensure accurate results.

FOR MORE INFORMATION

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Product release testing for endotoxin may be tested either prior to or after sterilization. The risk in testing prior to sterilization comes from potential changes in Gram-negative bioburden levels between the time of testing and the time of sterilization that consequently could impact endotoxin levels. Risk may be increased in samples containing water or nutrients. It is important to document this risk assessment if testing endotoxin prior to terminal sterilization to ensure patient safety.

There are different methods of analysis that can be performed based on modifications that are made to the TAL/LAL reagent. The selected method of analysis for endotoxin testing must be validated to ensure there is no inhibition or enhancement.

For all methodologies, sample positive product controls (PPCs) are run to measure potential inhibition or enhancement in the sample extraction solution. There may also be additional QC requirements such as test replicates or standard curve requirements (R2 values, CV%, Y-intercept, etc.).

These methods are detailed below:

Gel clot

The gel clot method of analysis is similar to how the horseshoe crabs immune system responds to endotoxin, by clotting. The method of analysis involves testing dilutions of the extract and determining whether the solution forms a solid gel in the presence of the horseshoe crab derived reagent. This method produces only a pass / fail result and is more subjective than the other methods as it is a very manual test. This test is now rarely used due to improved methods being available.

Kinetic Turbidimetric

The turbidimetric method of analysis utilizes spectrophotometry to measure the intensity/absorbance of light passing through a sample solution. If endotoxin is present in this method, the horseshoe crab derived reagent produces a clotting reaction that produces a solid mass in the solution. The cloudiness or turbidity of the sample affects the amount of light that can pass through the sample and correlates to the amount of endotoxin in the sample (established using a standard curve of known endotoxin concentrations). Time taken to reach a specific turbidity level is also considered in the measurement.

The Kinetic turbidimetric method is not suitable for samples with color, turbidity, or high viscosity.

Kinetic Chromogenic

The chromogenic method of analysis uses a chemically induced color change as a measurement of endotoxin. Like the turbidimetric method, it utilizes spectrophotometric measurement at an absorbance wavelength designated for the colorant (p-nitroaniline). The chromogenic method utilizes initial portions of the endotoxin LAL reaction-cascade to activate an enzyme causing the release of p-nitroaniline from the synthetic material yielding the yellow pigment. The amount of the yellow colorant in a sample is derived from the standard curve to obtain a quantitative result.

Photometric methods of analysis (Turbidimetric and Chromogenic) are not suitable for samples with absorbance (pigment) at the measured wavelength used for analysis.

New Technologies

New recombinant technologies have been developed to reduce the need for harvesting horseshoe crab blood for this testing. These technologies currently require additional validation work and submission to regulatory bodies, as alternative methods for use in product release testing.

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REFERENCES

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