Endotoxin Detection Part X:

Moving Forward with Accurate and Sustainable Endotoxin Testing to Meet Global Standards

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Endotoxin Detection Part X

Moving Forward with Accurate and Sustainable Endotoxin Testing to Meet Global Standards

A Note from the Editor

Welcome to our tenth supplement on Endotoxin Detection.

As we move through the third year of the COVID-19 pandemic the global pharmaceutical industry continues to bring new vaccines to market to combat the ever-evolving strains of this disease. We have also seen new treatments being developed to help those infected recover from this virus.

These products would not be possible without the combined efforts of manufacturers, technology suppliers, and government.

Yet, there is always more to be done.

As witnessed through recent news reports, contamination of products can lead to both drug and healthcare shortages. Factor in global supply chain issues, and the need to ensure products are free from any sort of contamination is critical before products leave the manufacturing facility.

As the market for biopharmaceuticals continues to expand – the industry's need to test for bacterial endotoxins also grows in importance.

With medicinal products and supply crucial to the global effort to combat not only COVID but a myriad of other diseases, there simply is no room for error when producing new products – consumer expectations and the need to ensure consumer confidence are at all-time high.

The goal of this supplement is to provide as much information as possible regarding current thinking and methodologies for endotoxin testing and removal. As indicated in the table of contents below there is much to be said on this topic – and we have enlisted the expertise of many of the industry's subject matter experts.

As you look through these articles, we hope you gain valuable insight and knowledge regarding this industry critical topic.

If you have any questions or comments, please contact us.

Thanks again for reading,

Mike Auerbach
Editor In Chef

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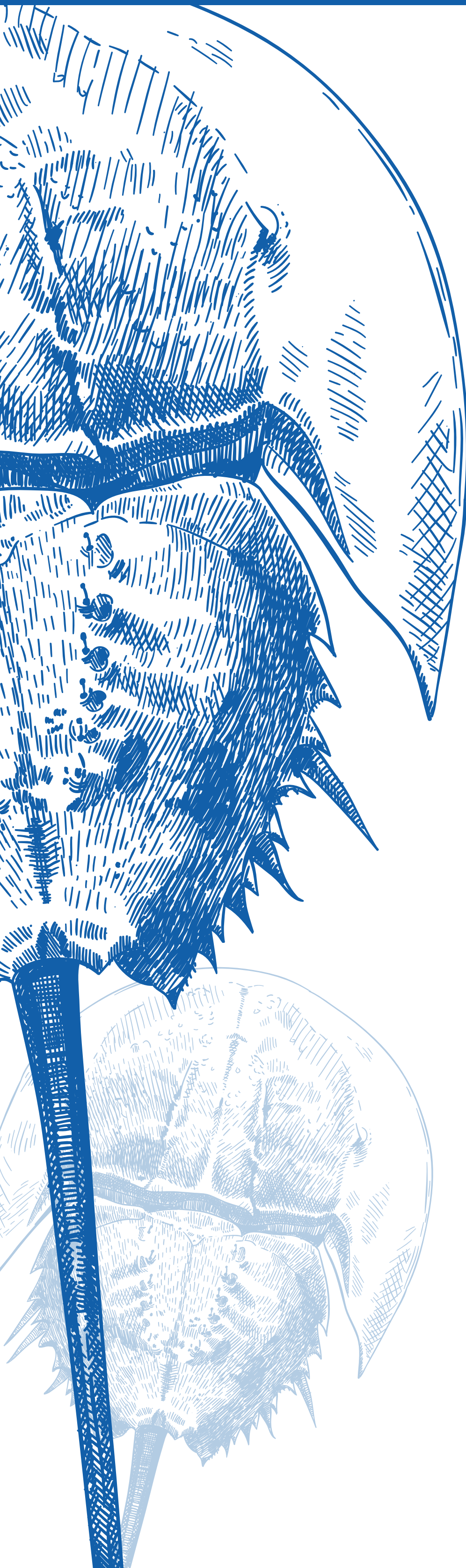
HORSESHOE CRABS AND THE BIOMEDICAL INDUSTRY...Know the Truth

Frequently Asked Questions



What makes a horseshoe crabs' blood so special?

Horseshoe crab blood carries factors that react to antigens found on and in gram-negative bacteria walls by forming a clot around it. The clot isolates the bacteria, and protects the crab from infection. The blood also begins a healing process similar to ours where we form a clot, a scab, and eventually wounds heal.

What makes LAL so important?

The LAL test is the most sensitive, accurate and cost-effective test on the market today to detect contaminating endotoxins. This test was first licensed by the FDA in the 1970s, and is now the gold standard. It can detect endotoxin in the parts per billion. That's like finding a grain of sand in an Olympic swimming pool. Prior to LAL, rabbits were used to test for endotoxins by injecting the rabbit with sample of the product being manufactured and waiting two or three days to see if the rabbit developed a fever. Hundreds of thousands of rabbits were required to be held and utilized this way. LAL based assays replaced this test with one that is more humane, more accurate, cost effective and can give results in a test tube, in about an hour. There are very few people you are likely to meet in your lifetime who have not benefited from a bacterial endotoxins test.

What types of things are tested with the blood?

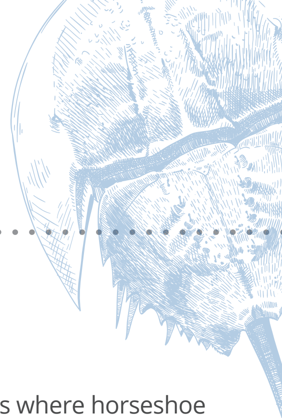
The FDA has mandated (it is the law) that all injectable or indwelling materials must be tested for endotoxin contamination before being released for sale. This is to protect the public from products that are not sufficiently free of materials that can make a patient ill from exposure to gram-negative cell wall material. If endotoxin enters your blood stream it can make you sick and possibly even kill you. So the test we manufacture is used for medical devices, such as knee replacements, stents, heart valves, intravenous solutions; and drugs and vaccines like childhood immunizations, insulin, flu vaccine and chemotherapy drugs to name a few. Anything injected or implanted into the human body must be free of endotoxin.

I have read somewhere crab blood is worth $15,000 a quart? Is that true?

Absolutely not. This is a myth sensationalized by some media. Manufacturing LAL which is made from the white blood cells of horseshoe crabs, is a complex process that is regulated by the FDA and must be done under extremely clean conditions. A typical LAL test costs less than $20. In terms of the impact it has had on human health and safety, it is safe to say it has saved many lives and is therefore priceless.

Where do the crabs you bleed come from?

Most of the crabs that come to our facility are from Massachusetts waters, Vineyard Sound, Nantucket Sound, and Buzzards Bay. Fisherman catch them a number of different ways but must follow strict regulations on size, number of crabs harvested, and quota management.



⊙ What can I do?

Water quality and human development are major threats to all fragile ecosystems such as the embayments where horseshoe crabs reproduce and grow. Do your part in mitigating the impact humans have on water quality and beach erosion. If you ever see a crab upside down on the beach, gently roll it over and return it to the water. And remember, the next time you or a loved one receives an injection, IV or implant, be sure to thank a horseshoe crab!

⊙ How does the process of bleeding the crabs work?

The process is very similar to when people donate blood. The crabs are checked for good health, placed in a very clean laboratory where we disinfect a portion of the shell, and carefully insert a sterile needle. We collect excess blood from a sinus in the dorsal aspect of the crab's body, just under the shell. The way the crabs are held limits the blood that can be harvested to the dorsal sinus. The majority of the blood which is in the gill areas is untouched. Studies have shown that the crabs tolerate this process very well and the overwhelming majority survives.

⊙ What threats face the horseshoe crabs today? Are they endangered?

Like any sea creature, horseshoe crabs are dependent on a suitable environment in which to live and reproduce. Water quality is an important factor as is having suitable beaches in which to lay their eggs. Fertilizers, septic systems, and other forms of pollution can greatly reduce the quality of water on which the crabs depend. Sea walls, rip-rap and jetties can manipulate the natural movement of sand on beaches and affect spawning habitat. Beach nourishment, the practice of bringing in truckloads of sand to beaches to replenish what's lost, or make them look nice, can bury millions of eggs before they hatch if not carefully timed. Crabs are also used as bait for conch and eels which is another source of man-made mortality. Crabs in the United States are regulated and monitored carefully. They are not endangered, in fact, in many areas populations are growing considerably. In other parts of the world, they are victims of pollution and humankind's development of coastal areas and are not so closely monitored.

⊙ What does ACC do to support conservation?

ACC has always promoted and practiced a catch and release fishery where the overwhelming majority of crabs survive the process of blood extraction. We work closely with fishermen and regulators to minimize the impact we may have on crab populations. ACC was instrumental in creating a minimum size limit for crabs to ensure only mature crabs are collected, and helps to keep a biomedical only fishery in Pleasant Bay, MA where all the crabs collected are released. We have supported conservation efforts that include the use of bait bags, decreased catch limits and a prohibitation on fishing for crabs around peak spawning periods. We also participate in the Massachusetts "rent a crab program" where crabs destined for use as bait are brought to our facility first. This helps to limit the overall impact on crabs, and is unique to Massachusetts. ACC takes part in the Atlantic States Marine Fisheries Commission (ASMFC) Horseshoe Crab advisory board where we helped develop the Best Management Practices (BMPs) for the industry. We also collect data for the regulators from every crab that enters our facility, which is invaluable to understanding population dynamics. Most recently ACC has implemented a one-of-a-kind sustainability project where we can create juvenile crabs *in-vitro* and release them to the wild.

⊙ What information should more people know about horseshoe crabs?

Horseshoe crabs and their ancestors have been on this planet for somewhere around 400 million years, and have survived mass extinctions. They are not harmful, don't sting, bite or try to do us any harm. Remember when you see a horseshoe crab shell washed up on the beach it is likely a molt, and not a dead crab. Crabs can only grow by shedding their shells and growing larger ones. Old shells are discarded and many beachcombers worry crabs are dying when they are really just growing up. Even as recently as the 1950s crabs were destroyed by the tens of thousands by people on Cape Cod and elsewhere fearing they were harmful to shellfish beds or for use as fertilizer and pig food. In fact, they are useful for shell fisherman by helping to till and keep sediment aerated. They are an important part of the international ecosystem.

Will a Proposed Reduction in Endotoxin Limits for Drugs and Biologics Improve Patient Safety?

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Any parenteral therapy theoretically carries a risk of bacterial endotoxins contamination which can result in a number of physiological responses in humans, including fever. It is common in modern clinical medicine for a single IV infusion of 250-1000 mL to contain several "piggybacked" therapies. If each component of the therapy (drug product, diluent, infusion fluids, syringes, transfer sets, etc.) were at its allowable endotoxins limit, patients would be at significant risk of a febrile response. However, there are no data in either peer reviewed clinical literature or the compliance literature, including adverse events and product recalls on FDA's website, to support this hypothesis, suggesting that this concern regarding additive endotoxins activity to unsafe levels is not a verifiable clinical issue. Despite the lack of data in the public domain, it is our understanding that a proposal has been advocated by a number of regulators to mitigate this hypothetical problem by reducing the endotoxins limits for drugs and biologicals by at least half.

We take a different view on the proposal to arbitrarily cut the endotoxin limits as we see no published or documented evidence of a problem. We believe that the continuously evolving science of endotoxins chemistry and their variable biological activity, the extensive use of the highly sensitive LAL test as a monitoring tool for manufacturing controls implemented for the mitigation of potential endotoxins contamination and the voluntary imposition of conservative in house limits.

The Problem

Two examples from current therapies serve to illustrate the hypothetical concern of additive endotoxins activity.

Example 1: COVID-19 Vaccine Package Insert

Table 1a. Product dose and Endotoxins Limit

	Adult (70 kg) Total Person Dose = 350 EU	Child (6 months, 7 kg) Total Person Dose = 35 EU
Drug Product Dose	30 mcg	3 mcg
Endotoxins Limit	11.6 EU/mcg	11.6 EU/mcg

Table 1b. Drug Product Administration

ENDOTOXINS CONTRIBUTOR	Adult (70 kg) Total Person Dose = 350 EU	Child (6 months, 7 kg) Total Person Dose = 35 EU
Drug dose	30 mcg/adult x 11.6 EU/mcg = 348 EU/adult	3 mcg/child x 11.6 EU/mcg = 34.8 EU/child
Syringe to withdraw and administer the dose	20 EU	20 EU
Total	368 EU	54.8 EU
Total (DP at half limit)	194 EU	37.4 EU

In Example 1, if the drug product and the syringe used to administer the dose are each at their maximum allowable limits, the "total person" endotoxins limit for both children and adults would be exceeded. If the limit for the drug product is halved, the "total person" endotoxins dose for a 6-month-old child is still exceeded.

Example 2: COVID-19 Combination Antibody Therapy Package Insert

Table 2a. Product dose and Endotoxins Limit

	Adult (70 kg) Total Person Dose = 350 EU	Child (6 months, 7 kg) Total Person Dose = 35 EU
Drug Product Dose	2100 mg/person	270 mg/person
Endotoxins Limit	0.16 EU/mg	0.12 EU/mg

Table 2b. Drug Product Administration

ENDOTOXINS CONTRIBUTOR	Adult (70 kg) Total Person Dose = 350 EU	Child (6 months, 7 kg) Total Person Dose = 35 EU
Drug Products	2100 mg x 0.16 EU/mg = 336 EU/adult	270 mg x 0.12 EU/mg = 32.4 EU/child
2 syringes for transfer of DP for infusion	40 EU	40 EU
1 sterile pre-filled infusion bag containing 0.9% NaCl (sizes 50-250 mL)*	25-125 EU (assuming the maximum LVP endotoxin limit of 0.5 EU/mL)	N/A
1 sterile empty infusion bag*	N/A	20 EU
1 infusion set*	20 EU	20 EU
1 in line or add on filter*	20 EU	20 EU
Total	441-541 EU	132 EU
Total (DP at half limit)	273-373 EU	116 EU

Note: Components with an asterisk (*) are required by the package insert for administration.

In Example 2, calculations indicate that if each of the required components was at its limit, the total body limit for both adults and a 6-month-old child would be exceeded. If the limits for the drug product are halved, the total endotoxins dose for both the adult and child is still exceeded.

Endotoxins and Threshold Pyrogenic Doses

Endotoxins are structural components of the outer membrane of most Gram-negative bacteria. The endotoxin complex affects membrane permeability, resistance to antibiotics, virulence, and recognition by the host immune system. In humans, endotoxins activity can initiate a febrile response that is mediated by the TLR4/MD2 complex (Molinaro, et al, 2015; Simpson and Trent, 2019). Although endotoxins in nature are often associated with outer membrane proteins and other membrane components, it is the lipopolysaccharide (LPS) portion of the endotoxins complex that is biologically active.

The LPS molecule can be divided into three parts: the strain-specific oligosaccharide side chain, the core oligosaccharide, and Lipid A. It is the Lipid A portion that anchors the molecule to the cell membrane and confers its biological activity. The backbone of the typical Lipid A portion of the molecule is an acylated di-phosphorylated diglucosamine, usually with 4-7 acyl chains of varying lengths. However, the specific chemistry of both the backbone and the length and number of acyl chains can differ dramatically among Gram-negative species (Trent, et al, 2006). Additionally, microorganisms can remodel their Lipid A chemistries as they adapt to changes or stresses in their environments (Raetz, et al, 2009; Simpson and Trent, 2019). Therefore, although the general structure of endotoxins is conserved, they can exhibit significant variability at the fine structure or molecular level, notably acyl chain number and length, and phosphorylation.

A foundational area of study during the development of the Bacterial Endotoxins Test (BET) was determination of the Threshold Pyrogenic Dose (TPD) of endotoxins. Greisman and Hornick (1969) were the first to observe that the TPD in rabbit and man were equal for three different purified LPS preparations. However, they found that it took 50-70 times as much *Pseudomonas* LPS (7 acyl chains) as *E. coli* LPS (6 acyl chains) to achieve a pyrogenic response. Several independent studies in rabbits during the 1980s allowed researchers to further define the TPD as 1 ng/kg, calculated as the lower 95% confidence limit of the average pyrogenic dose of purified *E. coli* LPS (Dabbah, et al (HIMA), 1980; Tsuji, et al, 1980; Weary and Pearson, 1982).

Recognizing that the potency (activity per ng) of LPS from a range of microorganisms is highly variable depending on its Lipid A structure, it was proposed that endotoxins be measured in terms of their activity rather than weight. The initial definition of activity in endotoxins units (EU) assigned an activity of 5 EU to 1 ng of the EC-2 *E. coli* standard. Therefore, the empirical TPD of 1 ng/kg for pyrogenicity is equivalent to an activity of 5 EU/kg.

Marlys Weary and co-workers (1982) compared the average pyrogenic dose and LAL test results for several purified LPS preparations. They observed that for purified LPS, the LAL test provides a 2-6X safety factor over the rabbit test for LPS with 6 acyl chains, and a 26-60X safety factor for LPS with 7 acyl chains, confirming the Greisman and Hornick findings. Loppenow and co-workers (1989) indicated that levels of Cytokine IL-1, one of the cytokines in humans released during the fever response, is also dependent on the number of LPS acyl chains, with six acyl chains being most active. These data demonstrate that the potency and therefore the pyrogenicity of and LPS are dependent on its chemical structure.

Environmental Endotoxins

Pearson and co-workers at Travenol (now Baxter) Laboratories and Donald Hochstein working at FDA compared rabbit pyrogenicity and LAL reactivity of "environmental" endotoxins found in raw materials, in process samples, finished biological products, and numerous water sources, including pharmaceutical water systems (Pearson, et al, 1982; Pearson, 1985; Hochstein, 1987). The taxonomic identification of the organisms contributing to this endotoxins activity were unknown, however the endotoxins clearly originated from Gram-negative bacteria autochthonous to the manufacturing environments, including manufacturing materials or source water

used during drug product manufacturing.

- Pearson, et al (1982) performed 8-rabbit tests on a total of 644 manufacturing samples where the endotoxins activity as measured by LAL exceeded 0.25 EU/mL. The researchers found that 99% of the samples exceeding an LAL result of 10 EU/kg samples passed the Rabbit Pyrogen Test (RPT), suggesting that the TPD may offer a "safety factor" particularly when measuring environmental endotoxins.
- In his 1985 study, Pearson looked at RPT results relative to titration of endotoxins detected by LAL in a bulk lot of the product Piromen, a preparation of *P. aeruginosa* polysaccharide. They found that the rabbit test passed at doses that measured 250 EU/kg and under. Notably, *Pseudomonas* LPS has 7 acyl chains.
- Hochstein published data in 1987 comparing the LAL and RPT on 333 lots representing four different finished biological products containing various levels of "environmental" endotoxins measured by the LAL test. He found that in final product the LAL test was on average significantly more sensitive than the RPT.

Taken together, these studies indicate that the utilization of the highly sensitive LAL test can provide a "safety margin" of between 10X and 50X over the RPT using "real world" products and materials containing endotoxins from autochthonous Gram-negative microorganisms.

Endotoxins Limits

The maximum human endotoxins exposure limit for a dose of drug product is calculated by multiplying the TPD (5 EU/kg for all administrations except for intrathecal) by the weight of the patient. The average weight of an adult in the US has been historically assumed to be 70 kg, making the adult "whole person" endotoxins limit equal to 350 EU (5 EU/kg x 70 kg). According to CDC growth charts, an average 6-month-old child weighs about 7 kg (CDC 2022), making the "whole person" endotoxins limit for a 6-month-old equal to 35 EU.

To assure patient safety relative to the empirically derived TPD, endotoxin limits are calculated for every parenteral drug or biologic administered. Per USP <85>, a chapter that is harmonized with the European and Japanese Pharmacopeia, a product-specific endotoxin limit is calculated using the formula:

$$K \div M$$

Where: K is a constant, which is the TPD of 5 EU/kg for all parenteral administrations other than intrathecal, which was assigned a limit of 0.2 EU/kg

M is the maximum dose of the product/kg/hr as defined in the package insert.

There are some caveats to this formula that relate to patient safety:

- If the pediatric dose of the product is higher on a per kilogram basis than the adult dose, the pediatric dose must be used as the denominator when calculating the endotoxin limit.
- If a product is administered for more than one hour, then M is adjusted to dose/hour.

Medical devices with product or patient contact, which include empty infusion bags, syringes, tubing sets, IV needles, and filters associated with the administration of parenteral drugs, were assigned an endotoxins limit of 20 EU/device regardless of where or how they're used. This assignment was based on the way in which transfusion and infusion devices were prepared for testing in rabbits.

Likewise, Large Volume Parenteral (LVP) preparations routinely used as infusion fluids such as saline, Ringer's lactate solution, or dextrose, were assigned a limit of 0.5 EU/mL, also based on the RPT.

The BET is an *in vitro* enzymatic assay, which has a level of analytical variability typical of biological assays. The original gel clot test was constrained by two parameters: test results were binary (either positive or negative) and the "standard" was a series of twofold dilutions. Given these constraints the resolution of the assay could be accurate only within a single two-fold dilution range (½x-2x or 50-200%). For photometric assays, this range is not indicative of assay resolution, expected assay variability or normal error in the assay, but rather represents limits on the range of interference that might arise in any one test sample due to the test sample matrix. It is possible that the proposal to arbitrarily cut endotoxins limits in half is based on misinterpretation of the 50-200% recovery of the Positive Product Control (PPC).

Is There a Problem?

The concern about potential risk to patients during therapy if the drug product and each of the medical devices used for administration are at their allowable endotoxins limit is clearly appropriate. However, arbitrarily changing the limit, as some have proposed, should require a verifiable or documented clinical risk.

- The TPD was originally determined based on a highly purified and highly potent purified LPS derived from the enteric microorganism, *E. coli*. Endotoxins activity in mammals is related to endotoxins chemistry. Enteric Gram-negative bacteria generally exhibit an LPS structure that is different in acyl chain number and length than the less potent non-fermenting Gram-negative bacteria that are more commonly observed in pharmaceutical manufacturing. Enteric or coliform bacteria are extremely rare process or product contaminants.
- "Environmental Endotoxins" are less potent per unit weight than purified LPS. One gram of Gram-negative cell walls has less LPS than one gram of purified LPS.
- While the aggregate "safety margin" afforded by the LAL test may not be easily quantified, the empirical evidence regarding the sensitivity of the test *vis a vis* the RPT coupled with 40 years of clinical use of products released using LAL suggest that there is little or no risk of all products being at their limits for any one administration of therapy, no matter how complex.

- It must be noted that the proposal requiring a 50% reduction in endotoxins activity also reduces the Maximum Valid Dilution by 50%, which may invalidate some existing method suitability studies requiring firms to re-execute costly and time-consuming experiments.

Since the advent of the LAL test in the 1970s and 1980s, manufacturers of parenteral drugs, biologicals and medical devices have taken advantage of a relatively inexpensive yet reliable and sensitive test to monitor the effectiveness of production and process controls intended to reduce or eliminate the risk of endotoxins contamination. Water systems have historically been a source of endotoxins in parenteral products (Seibert, 1923; Seibert, 1925). Although design and engineering of water systems have improved dramatically, the generation and distribution of this ubiquitous raw material are still monitored extensively using the LAL test. Manufacturing materials, particularly those derived from natural sources, are assigned endotoxin limits based on their use (e.g. API, excipient, etc.) and are tested for endotoxins activity prior to use. The identification and routine monitoring of critical control points that can either introduce or reduce endotoxins contamination have provided assurance that processes are consistently "cleaner" than the calculated endotoxins limit would allow. In addition, the voluntary imposition of in-house action/alert/release limits that are generally much lower than the calculated endotoxins limit has assured that products are safe as defined by the TPD.

Current limit-setting strategies based on dose have served patient safety well since first published by FDA in 1983 (Federal Register, 1983). To adjust a product's endotoxins limit to account for endotoxins contribution from co-administered products and delivery devices requires an estimation of the type, number, dosing, time of administration and the endotoxins content of the co-administered products. That number cannot be known by a manufacturer *a priori*. Because of the implementation of prudent risk mitigation measures by manufacturers, the risk of each component of an infinite numbers of combinations of drugs and administration devices being at their allowable endotoxins limits is virtually non-existent. The only estimate of endotoxins contribution would be the acceptance limit, as found in the product monograph or as calculated according to USP <85> or <161>.

The authors maintain that endotoxins science and proactive control of endotoxins in the pharmaceutical and medical device industries have resulted in substantial risk abatement relative to endotoxins contamination. The concern of increased patient risk due to multiple components being at their limits, however well intentioned, is not a documented problem making the arbitrary reduction of endotoxin limits unnecessary.

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Development and Qualification Factors for Endotoxin Removal from Proteins Using Chromatographic Columns

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Introduction

Purification is a necessary phase in pharmaceutical drug manufacturing in order to eliminate unwanted materials that can be hazardous or which will compromise drug efficacy. This is of particular concern with protein-based drug products, where impurities include closely related non-functional proteins. To achieve the desired purification, attention needs to be paid to controlling a range of factors including temperature, time, pH, salt concentration, protein concentration, surface interaction and mechanical stresses. As part of purification process, contaminants also need to be removed; examples of contaminants include viruses and bacterial endotoxin,[1] and the risk posed by these will depend upon the source material and/or the type of end product. The presence of endotoxin, at a sufficient threshold, can cause patient harm in terms of a pyrogenic reaction[2] or endotoxic (septic) shock,[3] especially given that most biological products are administered by injection and many protein-based pharmaceutical product doses are low. Some proteinaceous products, especially those derived from starting bacterial cultures, are especially at risk from endotoxins; and proteinaceous products in general present a risk not least because endotoxins have the tendency to bind and form complexes with many types of proteins. Therefore, biomanufacturing steps must seek to both minimize endotoxin contamination through a contamination control strategy and remove ('clear') any endotoxins present at appropriate stages.[4] Even where there is no therapeutic necessity to remove endotoxin, the presence of endotoxin can cause false readings in cell-based assays, providing a further reason to remove endotoxin from the purified product.

Within biopharmaceutical manufacturing, column chromatography is a common method of protein purification[5] (the biophysical technique enables the separation, identification, and purification of proteins based on their size, shape, net charge, stationary phase used, and binding capacity).[6] The principle of chromatography is based on two phases: the mobile phase

which drives the sample and the stationary phase which allows its purification. The main separation techniques are ion exchange, surface adsorption, partition, and size exclusion.[7] Commonalities between these chromatography methods is that they each use a resin (the solid phase) with special chemical properties, which are held within a glass cylinder (the "column").

The commonly used chromatography methods to remove endotoxin are:[8,9]

1. Anion exchange chromatography using the negative net charge of endotoxin to bind endotoxin to an anion exchange resin (or the reverse, where positively charged proteins are bound to the resin).
2. The use of adsorbents to facilitate adsorption of endotoxin to matrix by electrostatic and hydrophobic interactions.

While ion exchange chromatography has traditionally been employed for endotoxin removal from protein solutions, greater success tends to be realized through the selection of affinity ligands. This article considers these methods as part of the development and qualification requirements that enable column chromatography to deliver endotoxin clearance as part of the protein purification step.[10]

Endotoxin Contamination

Many proteins will be contaminated with endotoxin, either due to the origin of the proteins (if derived from bacteria like *Escherichia coli*), variables within the manufacturing process (addition of excipients and water), or from control breakdown (the risks presented by wet equipment or uncontrolled process hold times, for example).[11] When endotoxin and protein come into contact an association is often formed primarily from electrostatic interactions although other mechanisms can occur, such as binding domains and hydrophobic interactions. These interactions can form various complexes and the bridges that form between proteins and endotoxin are very stable, making the disassociation difficult.

The weight of endotoxin capable of causing patient harm can be as low as 0.1 nanograms (depending upon the route of administration), and this quantity can be produced by around 100,000 Gram-negative bacteria,[12,13] a relatively low cell number that emphasizes the importance of manufacturing controls. Naturally occurring endotoxin is primarily lipopolysaccharide (LPS), in various aggregate forms with a typical molar mass of 10 kilo Daltons (kDa). It is a component of LPS - the hydrophobic lipid group covalently bound to a long complex polysaccharide tail (referred to as Lipid A) - that is responsible for the adverse physiological reactions that occur within the bloodstream of mammals where endotoxin induces the secretion of pro-inflammatory cytokines (such as interleukin-1 by macrophages) which triggers systemic inflammation by stimulating the hypothalamus to produce prostaglandins, which increases the body's core temperature, culminating in a fever.[10] The challenge presented by endotoxin is that it is indestructible relative to the product, in that applying methods that are capable of inactivating endotoxin – heat or chemical - will destroy proteins first. Therefore, the focus is with endotoxin removal from the product matrix by separating protein and endotoxin.[14] In the context of this article, since endotoxin is negatively charged at pH above 2 (LPS, with the Lipid-A component, is partially phosphorylated), the basis of removal is through the use of positively charged chromatography (anion exchange) or with absorption as the result of affinity chromatography, or sometimes, a synergy of the two removal processes.[15]

Ion Exchange Chromatography

Ion exchange chromatography separates ionizable molecules based on their total charge. The method technique enables the separation of similar types of molecules. The charge carried by the molecule of interest can be readily manipulated by changing buffer pH. With endotoxin, the principle is based on endotoxin, at most pH ranges saves extreme acidity, being negatively charged and the resin used in anion exchange chromatography being positively charged. Where the protein is also positively charged, the process of movement through the chromatography column will pull endotoxin away from the protein and retain it on or within the resin. Clearance factors of around 5-logs can be achieved with ion exchange chromatography, although only from high starting concentrations of endotoxin.

Depending on the process, the charge effect can also be used with certain filters where a positively charged membrane that electrostatically attracts and/or retains endotoxins,[16] although this is not practical for all applications not least due to yield losses.

Affinity Chromatography and Endotoxin Removal Resins

The most effective way to remove endotoxins from proteins to be purified is with the use of a suitable column and an endotoxin removal resin, one that also delivers the desired level of purification where the protein of interest is recovered in the flow-through. Certain chromatography resins have an affinity for endotoxin, by being able to absorb endotoxin from a solution, given sufficient contact time, through both ionic and hydrophobic interactions. Many resins come in the form of spherical particles.[17] The properties of the resin and its efficiency to achieve endotoxin absorption is a product of the resin's affinity ligands. Ligands are ions or molecules that bond to a central metal atom and function as electron pair donors, with the central atom functioning as an electron pair acceptor.[18] Hence, electrostatic interaction and hydrophobic intermolecular interaction are the principal interactions in delivering the adsorption of endotoxin.[19]

Assessing resin efficiency

Selecting the optimal resin for both achieving protein purification and removing endotoxin presents a critical choice for the development and qualification of the pharmaceutical manufacturing process. Important criteria to establish, and to verify through validation are presented below:[20-25]

- The resin ligand should be LPS-selective and possess a combination of cationic properties and hydrophobic properties. LPS binding should be non-specific.
  - The resin should function under the required physiological conditions.
- The desired and expected level of endotoxin removal (which is typically expressed as a logarithmic reduction). In terms of the desired levels, this would typically be <5 EU/mL (based on the critical quality attribute of the finished product). The Endotoxin Unit (EU) is the measure of biological activity, enabling endotoxins of different molecular weights from different bacterial species to be compared.
  - This may require an understanding of the potential challenge levels and consideration of any controls upstream of the column, since the efficiency of endotoxin removal will be part-dependent upon the challenge level (in that the higher the endotoxin challenge, the less efficient the removal process will be).
- The capacity for endotoxin removal relative to the maximum amount of pharmaceutical ingredients to be processed.
  - The ionic strength of the material passed through the column will affect process efficiency.
- The process time required, which is based on column flow rates and the contact time required between the processed material and the resin in order to remove the required level of endotoxin. There is a trade-off required between the use of a bead-based chromatography resin, which has superior absorbency, and a membrane based one that achieves better flow but is less effective at endotoxin capture.
  - The adsorption of endotoxin takes place mainly at the outer surface of adsorber particles. While adsorption can occur within the resin, the contact times required, as shown by long uptake adsorption curves from experiments, are often too long in terms of process efficiency.
- Assessing the affinity of the endotoxin for the protein and how this can be modified by factors such as temperature, pH, detergents (surfactants), solvents and denaturants.
  - Where pH can be altered, maximum endotoxin deactivation occurs at acidic pH below isoelectric point.
- The number of times the resin can be re-used, including understanding the point at which endotoxin removal efficiency decreases. This consideration will need to extend to the column regeneration process.
- To demonstrate that the resin does not introduce any impurities.
- That the resin is easy to pack into the column.

The points listed above should form part of method development and be verified as part of process validation. Some of the points will need to be developed in conjunction with the column supplier, given that the introduction of large quantities of endotoxin, although needed to demonstrate column efficiency, into the production process carries considerable risk (typically 10,000 or 100,000 EU/mL is required). Such

a study should use the actual material to be processed (or a suitable placebo), although experiments can be scaled down provided the laboratory study is appropriately representative of what will become the scaled-up process. Once this assessment is completed, the focus of the user will be with maintaining control (such as controlling the time point for resin changes and practicing column depyrogenation) and in-process monitoring (where a sample is taken from each batch processed through the column to verify absence, or sufficiently low levels, of endotoxin using an endotoxin test method, such as the *Limulus* Amebocyte Lysate (LAL) assay).[26] Batch related in process monitoring of intermediate product provides an important batch-by-batch assessment of the continuation of controls.

In addition, the ability of the resin to achieve the desired level of protein purification must also be met during pilot studies, otherwise the exercise becomes uneconomical for scale-up. Common types of resins include affinity adsorbents based on the antibiotic polymyxin-B and sodium deoxycholate; poly(ethyleneimine); more novel resins are based on lysine (poly-L-lysine) or polycationic ligands.[27,28] Generally, endotoxin will elute at pH 8 around 500 mM NaCl. The important element is the surface modification of the resin so that it has the highest possible affinity to endotoxin, meaning that lipopolysaccharide effectively binds to the resin.[29] The process can sometimes be enhanced through the presence of a surfactant, either as a pre- or post-column processing stage (a phase separation method). For example, the addition of surfactant Triton X-114 (a nonionic surfactant that has a hydrophilic polyethylene oxide chain) and subsequent incubation promotes the association with lipid A.[10,30] When followed by a refrigeration step, this can allow the surfactant to gelatinize, enabling its removal with the associated endotoxin. The use of a surfactant can be doubly useful, when seeking the simultaneous clearance of metal chelates. Additionally, Triton X-114 can be used for the washing of columns as part of the column decontamination wash.[31]

Development and Qualification Complications

It is important to demonstrate removal of endotoxin from protein since methods of endotoxin detection are limited in their ability to detect both low levels of endotoxin (at picogram levels) and some protein-endotoxin interactions can lead to masking, where endotoxin may be present but not detectable using conventional endotoxin test methods like LAL.

This places an importance on demonstrating endotoxin removal through developmental study. However, the removal of endotoxins, particularly with affinity chromatography, can be challenging when endotoxins are strongly associated with specific labile biomolecules. Complications to the endotoxin removal process also occur through the tendency of endotoxin to form micellular (cellular aggregate) or vesicular structures (as lamellar, cubic or hexagonal inverted arrangements). With size exclusion resins, for example, the relatively large size of the micellular form of endotoxin can cause the molecule to function like a larger biological molecule. Furthermore, with anion-exchange chromatography, the negative charge of endotoxin can lead to interactions with anion exchange resins, resulting in the co-purification of endotoxins with the other biological molecules.[32]

With ion exchange, the technique is optimal for positively charged proteins; whereas, negatively charged proteins pose the problem of product loss (proteins take on different charges based on pH). Thus, the success of affinity and ion-exchange chromatography in separating endotoxin from proteins is affected by the properties of the target protein. To overcome this, instead of binding the endotoxins to positively charged surfaces and allowing protein solutions to flow through, the process is modified by using cation exchangers to bind the proteins to negatively charged surfaces and allowing endotoxins to flow through and then recovering the protein. This works most effectively at pH 4, although this is less effective in terms of yield recovery.

Column Depyrogenation

Even where resins are used that are effective at capturing endotoxin, removing endotoxin from a chromatography column is an important step since sufficient build-up of endotoxin will lead to contamination of subsequently processed materials. The process of inactivating and removing endotoxin from a column needs to be undertaken periodically (as defined by the facility contamination control strategy) and the process takes time, which needs to be built into the facility processing schedule. The most common way to achieve depyrogenation of the column is to subject the column and matrix to a wash with sodium hydroxide (NaOH). The contact time and molarity of the sodium hydroxide will be dependent upon the type of resin and the expected level of contamination (which process validation studies can provide data upon). Common protocols for depyrogenation include overnight in 0.5 M NaOH or 4 hours in 1.0 M NaOH[33] (although endotoxin inactivation may require a contact time of more than 12 hours,[34] hence this becomes an important part of the equipment and process validation. Most qualifications seek to achieve a six-log reduction in an endotoxin challenge). Following the depyrogenation, the NaOH needs to be removed from the column, which requires large quantities of Water for Injection and optimization to ensure that all valves and lines have been effectively flushed before starting the next purification process.

Conclusion

The removal of endotoxin from proteins as part of the purification step is a critical control process in biopharmaceutical manufacturing since it is the primary, and sometimes only, means to remove endotoxin. The two principal methods to achieve this are ion exchange and adsorption. These methods have their own advantages and disadvantages as well as being more suited to the purification of

certain proteins. Depending on the desired outcome, a two-phased approach is sometimes required.

While column chromatography can help with decreasing the level of endotoxin present, no single or dual method should be relied upon, and minimizing endotoxin should be considered as part of the contamination control strategy. The efficiency of any removal process is dependent upon the challenge, therefore a risk-based approach to contamination control should foremost be centered on reducing the possibility of endotoxin presence upstream of the column. An additional control requirement is with avoiding subsequent recontamination of the purified product as it continues its path downstream.

As well as control, assessing intermediate material post-purification for endotoxin serves as an important verification step to ensure that the process is working as designed (albeit the endotoxin detection method sensitivity limitations acknowledged). It is also important that controls are in place and working to prevent any recontamination of the protein by endotoxin through downstream processing, as might occur from wet equipment or from the addition of contaminated excipients. Throughout the process, the use of intermediate manufacturing endotoxin monitoring can also partly offset other concerns being grappled with by industry: the issue of finished product low endotoxin recovery.[35]

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Specializing in chromogenic and turbidimetric reagent technologies, Associates of Cape Cod, Inc. (ACC) has been a global leader in endotoxin and (1→3)-ß-D-glucans detection products and services for nearly 50 years. ACC pioneered LAL testing methodology and was the first FDA licensed company to manufacture LAL reagents, and throughout the years has grown to be an internationally recognized leader in endotoxin detection.

Last year, we were very excited to introduce another first when we launched the first and only commercially available sustainable BET Recombinant Cascade Reagent (rCR), PyroSmart NextGen®. PyroSmart NextGen® is completely horseshoe crab blood free and unlike first generation recombinant BET reagents (rFC), PyroSmart NextGen® is the only one that uses the same LAL cascade as traditional LAL reagents, while eliminating the potential for 1,3-b-D-glucans. This eliminates the need to change test methods and purchase new specialized instrumentation as required by first generation rFC recombinant reagents. Simply put… same Instrument, same preparation steps, same method. **Keep your Method… Make an Impact!**

Our worldwide headquarters are located in East Falmouth, Massachusetts. With a dedication to quality, ACC is certified to I.S. EN ISO 13485:2016 and ISO 13485:2016. We are FDA Inspected and operate DEA Licensed and CLIA-certified laboratories. Our endotoxin detection reagents, instruments and software are used within the Pharmaceutical, Medical-Device, Biotechnology, Compounding Pharmacy and Dialysis industries for quality control, product release and research. Our reagents are FDA licensed and can be used for testing in compliance with USP, EP and JP bacterial endotoxin test chapters, and our software is 21 CFR Part 11 Compliant.

ACC also operates a Contract Test Services (CTS) Laboratory which has specialized in testing for endotoxin and glucan contamination for over 40 years. Our CTS laboratory is GMP compliant, ISO registered and DEA licensed and is capable of handling all controlled drug substances except those included in Schedule 1. All testing services can be performed to FDA, USP, EP and/or JP regulatory guidelines. In addition to routine testing, our CTS Laboratory will customize endotoxin testing, troubleshoot difficult samples, develop and/or transfer LAL test methods, design and produce custom depyrogenation controls for oven validation and perform Low Endotoxin Recovery (LER) studies/protocols.

ACC also offers a clinical diagnostic product line – Fungitell® and operates a CLIA-certified laboratory specializing in (1→3)-ß-D-glucans analysis to support the diagnosis of Invasive Fungal Disease (IFD).

Opinion: Reflections on Testing Medical Devices—Is it Time for an Update?

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Introduction

There was a time when medical devices were largely pieces of extruded or molded plastic. In fact, until recently, the name of USP chapter <161> describing pyrogen and endotoxin testing of medical devices was entitled, "Transfusion and Infusion Assemblies and Other Medical Devices."

Since that time, many innovative companies have developed and marketed remarkable products that are classified as medical devices (current term is MedTech), but are far different than the plastics that largely defined devices 35 years ago. Medical devices now include but are not limited to: new orthopedic replacements, medicated irrigation solutions, wound dressings that may include regenerative cell technologies, wound debridement treatments, "artificial" as well as autologous/allogeneic/xenogeneic skin grafts and 3-D printed devices. Do the test preparation procedures and limits described in <161> still apply, or is it time to re-think the methodology to be more inclusive of contemporary products and their intended uses? In my opinion, it's time to reassess.

Preparation of Medical Devices for Testing

The genesis of the endotoxin limit for medical devices was USP <151>, "Pyrogen Test " also known as the Rabbit Pyrogen Test or RPT. Briefly, the sample size for medical devices is generally 10 units. Each device is "extracted" or "rinsed" with 40mL of Water for Injection (WFI). 40mL was chosen as it was a sufficient volume to extract most transfusion and infusion devices, and the pooled extract provided sufficient volume for a three rabbit pyrogen test, and if necessary an additional five rabbits. After an hour of contact with the WFI, the extracts were pooled, made isotonic, and subsequently injected into the rabbit at a dose of 10mL/kg.

USP <161> was revised in 2017 to include references to medical devices other than transfusion/infusion assemblies including liquid medical devices (e.g. dialysate), gels and bone matrices. But the update lacked specific and practical guidance on the application of the chapter's content to these "new" devices. How does one extract a gel or a regenerative cell treatment or a wound treatment that is not a dressing? In fact, these new devices have often been prepared for testing as if they were drugs in accordance with USP <85>, but with the assigned limit of 20 EU/device rather than the maximum limit for a dose of a drug product of 350EU/person/hr (5 EU/kg/hr) x (70kg/adult).

Assignment of the Endotoxin Limit for Medical Devices

In the 1970s and 1980s, when LAL was proposed as an alternative to the RPT, the same sample/extraction scheme used to prepare devices for the RPT was maintained. But FDA's 1987 "Guideline on Validation of an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products and Medical Devices" (now retired and referred to as "the Guideline") imposed an endotoxin limit on the extract of 0.5 EU/mL, which is the endotoxin limit for Large Volume Parenteral (LVP) products other than WFI.

$$(40\text{mL of extract/device}) \times (0.5\ \text{EU/mL}) = 20\ \text{EU/device}$$

The Guideline recognized that devices come in all sizes, so if the extraction volume changed, the endotoxin limit/mL of the extract could be adjusted accordingly such that the limit for the device remained at 20 EU. For example, if the extraction volume was 10mL rather than 40mL, the limit for the extract would be 2 EU/mL. A general formula for calculating the EU/mL of device extract is:

$$(K \times N) \div V$$

Where K = Endotoxin Limit/device (20 EU for devices except for those used intrathecally, where the limit is 2.15 EU/device)

N = number of devices tested
V = total volume of the extract

Pooling the extracts from individual devices dilutes endotoxins that could be contributed by any one device. The Guideline acknowledged that *"In the worst case situation, all endotoxin present in the combined rinsings of 10 devices could have come from just one device."* In practical terms, if 9 of the 10 devices have undetectable levels of endotoxins activity, the 10th can have up to 200 EU/device. Is 200 EU/device the real limit?

Yes, it would appear so. However, a number of researchers representing lysate manufacturers, FDA and industry have attempted over the years to perform spike/recovery studies on a variety of devices and found that the extraction efficiency of the standard device preparation was generally less than 100%. (Ross and Twohy 1985; Twohy and Duran, 1986; Roslansky, et al, 1991; Berzofsky, et al, 1994). Not only was recovery low, but was dependent on a number of variables including the materials of construction of the device, the type of endotoxin (LPS vs "natural"), the total activity level of the inoculum, the composition of the extraction solution (water vs water plus dispersing agents), sonication and vortexing to name a few. The results of these studies as summarized by an AAMI Task force in 2004, suggested that the tenfold safety factor afforded by the 20 EU/device limit was sufficient to obviate the need for routine spike/recovery studies on devices and it was also sufficient to assure the safety of medical devices (Bryans, et al, 2004).

Applying Device Endotoxin Limits

Dialysis solutions have been assigned an endotoxins limit of 0.5 EU/mL, which is the same as the LVP (but not WFI) endotoxin limit. The advent of other unique devices raises questions regarding the application of endotoxin limits to these innovative products:

Example 1.

A manufacturer of skin grafts makes units in four different sizes. How is the endotoxin limit applied? To the largest graft? To the smallest graft? Per cm^2 of the graft? Per the maximum area of graft(s) that a patient can receive in one hour?

Example 2.

A wound care product is administered as a lotion, salve, gel or suspension in amounts ("doses") that are relative to the extent of the tissue damage. How does one "extract" these devices? Does it make sense to think of these products as drugs rather than devices? If so, how does one consider the "dose" of these types of medical devices? Per maximum application in an hour?

Example 3.

Devices that are assayed as drugs are prepared per <85>, meaning that they are subject to suitability (inhibition/enhancement) studies. For these devices, unlike the standard plastic medical devices, interference is mitigated and the PPC must be recovered as outlined in <85>. Is it reasonable to increase the endotoxin limits for these devices to 200EU/device?

Example 4.

Many newer medical devices utilize materials from natural products (e.g. alginates) that often contain endotoxins and/or glucans. These interferences are mitigated during suitability by <85>, but the 20 EU limit is often a constraint to mitigation, as calculated MVDs against a 20 EU/device limit (regardless of "dose") may prove difficult for sample preparation.

Example 5.

The Threshold Pyrogenic Dose is 5 EU/kg. For devices intended to be used in infants (e.g. a 3.5 kg infant), if a device is at its 20 EU max (or more given the dilution factor of pooling extracts), it would deliver almost 6 EU/kg, which for a drug product would be a failure. Should we be focusing more on the target patient population and intended use of the device?

Summary

In summary, innovative companies today are making medical devices that 35 years ago would have been the stuff of science fiction. Yet, our test method and endotoxin limit for medical devices has remained the same for the last 35 years. Should limits or testing methods change? Maybe or maybe not, but I would suggest that it is time to reconsider methods and limits in light of the new universe of medical devices and make decisions for safety and testing that are consistent with the composition of 2022 products and their intended use.

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AN INTERVIEW WITH...

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__Veronika Wills__ has been working in the endotoxin industry for the past 15 years and currently manages the Technical Services department at Associates of Cape Cod, Inc.

Veronika is a specialist on testing complex samples, method development, regulatory aspects of endotoxin testing, development and delivery of educational and technical contents.

She attends and speaks frequently at scientific conferences and seminars globally and is an established expert on endotoxin testing. Veronika holds a master's degree in Biochemical Engineering from the Institute of Chemical Technology in Prague, Czech Republic.

What are the current accepted test methods for BET?

The methods that are currently defined in the harmonized Bacterial Endotoxins Testing (BET) chapters are all based on the use of animal derived lysates of amebocytes (either Limulus amebocyte lysate (LAL) or Tachypleus amebocyte lysate (TAL)). These biological methods are broken down into two groups: gel clot and photometric methods (where photometric methods can be done using either turbidimetric or chromogenic reagent). In addition, the European Pharmacopeias added another chapter describing BET – this is chapter 2.6.32 which specifically describes testing for endotoxin using recombinant Factor C reagents (rFC). However, it is of note, that all EP monographs continue to refer to chapter 2.6.14 (the BET chapter using LAL reagents).

Tell us about emerging test methods such as Recombinant Factor C (rFC) and Recombinant Cascade Reagent (rCR). What are the benefits of rCR?

From the practical standpoint, the recombinant methods bring many significant advantages to the pharmaceutical industry, the Quality Control laboratories in particular: improved specificity and increased reproducibility of the signal of the endotoxin response. For many global companies it is also vitally important that neither recombinant reagent requires the harvest of live animals for the collection of the raw material, thus the reagents are and will be sustainably manufactured for years to come. In addition, the recombinant Cascade Reagent inherently provides other advantages to the end user – because the rCR is a chromogenic method, just like one of the LAL-based methods. This means that both LAL chromogenic and rCR chromogenic methods rely on the use of the cascade enzymes from the LAL reagent where the pro-clotting enzyme reacts with the chromogenic substrate to trigger the increase in measured absorbance at 405nm. This makes assessment and feasibility studies of the rCR extremely user friendly as the end user will use the same preparation steps, software protocol and instrument to perform the recombinant test.

What needs to be done in order for global regulatory bodies to incorporate rCR test methods into their technical standards?

All the pharmacopeias base the implementation of new, improved, rapid methods into the standards based on unbiased data. The data must be available from a statistically significant data set where equivalency of the new (thus alternative) method is under evaluation compared to the benchmark method. For the recombinant reagents for BET, this is referred to as comparability data in the US. This means that the recombinant methods must be first used within the industry to help generate a significant data set on relevant samples. This process is fairly time-consuming, but we believe that it is well under way. ACC has recently shared a large-scale water comparability study performed on over 80 samples to study the equivalency of rCR to LAL but also LAL to LAL results. The equivalency of rCR to LAL was shown higher than equivalency of LAL to LAL results.

Based on this information, what is the best way to move forward to ensure the rCR test method becomes the preferred test method?

We believe that the best way to move forward is early implementation of rCR on in-process samples (such as in-process water) which are not technically considered to be compendial samples. Testing in-process water samples by both methods (rCR and LAL) during a pre-defined period of time will allow generating onsite data studying the equivalency of the reagents.

Can you tell us about the PyroSmart NextGen® system including its method validation and how its features and benefits are moving rCR testing forward?

PyroSmart NextGen® reagent, as the first commercial GMP-manufactured recombinant cascade reagent for BET has many advantages that were confirmed and validated by first adopters. PyroSmart NextGen®, as a chromogenic reagent, provides a smooth transition from either photometric LAL reagent on any absorbance reader and software (whether it is a plate reader or tube reader), while utilizing the same standard operating procedures. It also provides the highest sensitivity available for the recombinant reagents 0.001 EU/mL in half the time of the LAL reagent. And it was found to be at least as suitable for testing a wide range of finished drug products of different types, or even more suitable than the LAL. Thus, thanks to the reproducibility and specificity, PyroSmart NextGen® can be used as a tool to troubleshooting BET assays.

European Pharmacopoeia Approach to Testing for Pyrogenicity

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Introduction

The texts of the European Pharmacopoeia (Ph. Eur.) play a major role in ensuring the quality of medicines in Europe. They consist in general chapters and monographs, which are mandatory quality standards ubiquitously applied by the licencing authorities of the 39 signatory countries of the European Pharmacopoeia Convention and the European Union, with the overall aim of protecting public health. The European Pharmacopoeia Commission, the decision-making body of the Ph. Eur., is responsible for the elaboration and maintenance of its content. The European Directorate for the Quality of Medicines & HealthCare (EDQM) is a directorate of the Council of Europe and is entrusted with publishing the Ph. Eur. and bringing these standards to its users.

It goes without saying that any official standards dealing with the quality of medicines must address the issue of potential contaminants in the products concerned. Medicinal products contaminated with pyrogenic substances and administered parenterally may cause adverse reactions ranging from fever to life-threatening shock-like symptoms. The aim of pyrogenicity testing is to limit, to acceptable levels, the risk of these adverse reactions happening.

In the Ph. Eur., medicinal products are tested for pyrogenic substances according to general chapter *2.6.8. Pyrogens*. The test consists of measuring the rise in body temperature induced in rabbits by the intravenous injection of a sterile solution of the substance to be examined. The chapter was first published in the Ph. Eur. in 1971 and is still prescribed in a large number of monographs and general chapters.

Endotoxins from gram-negative bacteria (lipopolysaccharides) are the most common cause of pyrogenic reactions induced by contaminated pharmaceutical products. The level of

bacterial endotoxins is verified using the procedures described in Ph. Eur. general chapters *2.6.14. Bacterial endotoxins* or *2.6.32. Test for bacterial endotoxins using recombinant factor C*, published for the first time, respectively in 1987 and 2020. These are the analytical methods most commonly used to address the pyrogenicity of medicinal products administered parenterally. They present the great advantage of avoiding the use of laboratory animals but the drawback of not detecting fever-inducing substances other than bacterial endotoxins.

There are, indeed, a small number of pyrogens that possess a different structure and that cannot be detected using the test for bacterial endotoxins. Such pyrogenic substances are detected using the procedures described in the general chapter *Monocyte-activation test (2.6.30)*. The monocyte-activation test is therefore an *in vitro* pyrogen test that has the advantage not only of avoiding the use of laboratory animals, but also of being able to detect any pyrogenic substance, i.e. both endotoxin and non-endotoxin pyrogens.

Replacement of the Rabbit Pyrogen Test

The Council of Europe's *European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes* was opened for signature in 1986. Since that time, the Ph. Eur. Commission and its experts have carried out a program of work committed to Replacing, Reducing and Refining (3Rs) the use of animals for test purposes. Achievements have been significant,[1] but there are still challenges ahead. The Convention is referred to in a number of Ph. Eur. texts, including chapter *2.6.8: "In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. Wherever possible and after product-specific validation, the pyrogen test is replaced by the monocyte- activation test (2.6.30)."* In spite of this explicit instruction to replace the rabbit pyrogen test (RPT) by its *in vitro* alternative, the animal test continues to be widely used.

At its annual conference in 2018,[2] the European Partnership for Alternative Approaches to Animal Testing (EPAA) reported on a survey performed among European companies and testing institutes that still routinely perform the RPT and found that there is little incentive to perform alternative testing when a pyrogen test is prescribed in a monograph. The regulatory burden linked with the change to the *in vitro* test was also mentioned.

Reading the Ph. Eur. texts only, users reported a potential discrepancy between monographs and EU Directive 2010/63/EU:[3]

"Article 4

Principle of replacement, reduction and refinement

1. *Member States shall ensure that, wherever possible, a scientifically satisfactory method or testing strategy, not entailing the use of live animals, shall be used instead of a procedure."*

"Article 13

Choice of methods

1. *Without prejudice to national legislation prohibiting certain types of methods, Member States shall ensure that a procedure is not carried out if another method or testing strategy for obtaining the result sought, not entailing the use of a live animal, is recognised under the legislation of the Union."*

According to Article 13 of the directive, the instruction given in chapter 2.6.8 – to use an alternative to the animal test – should be applied systematically, but this is not done in practice.

In view of the situation, the complete removal of the RPT from the Ph. Eur. is necessary if the aim is to move towards the exclusive use of *in vitro* tests for the control of pyrogens.

Currently, chapter 2.6.8 is prescribed in 59 texts of the Ph. Eur.: three general monographs (including 2034 *Substances for pharmaceutical use*), three dosage form monographs (including *0520 Parenteral preparations*), three general chapters and 50 individual monographs, covering such diverse products as antibiotics, human vaccines and blood products. In June 2021, the Ph. Eur. Commission endorsed the strategy for the replacement of *2.6.8* in all of these 59 texts.[4] A new general chapter *5.1.13. Pyrogenicity* will be introduced in the Ph. Eur., which will provide guidance to help users decide on their own approach to pyrogenicity testing, based on a risk assessment: depending on the potential presence of non-endotoxin pyrogens, the user will have the choice between an *in vitro* pyrogen test or a test for bacterial endotoxins. Suppressed from all texts of the Ph. Eur., chapter 2.6.8 will no longer be an option and will ultimately be suppressed from the Ph. Eur. The whole exercise will take approximately 5 years and stakeholders will be consulted via the usual channels with, in 2023, the chance to consult all proposed revisions and the new general chapter *5.1.13* – currently under preparation – in Pharmeuropa online[5] and to comment as necessary.

Recombinant Factor C

The test for bacterial endotoxins uses, as its main reagent, the amoebocyte lysate from an animal, the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). Discussions among Ph. Eur. experts on the use of a synthetic alternative to this natural reagent, recombinant factor C (rFC), have been ongoing since 2006. It took over a decade to collect sufficient data for the method using the synthetic reagent to be described in the Ph. Eur. A major breakthrough came on July 1, 2020 with the publication of general chapter *2.6.32. Test for bacterial endotoxins using recombinant factor C* in the Ph. Eur.,[6] giving an official status to the procedure using the recombinant reagent. In January 2021, the procedure entered official use as a Ph. Eur. method. In April 2021, the EDQM broadcast a webinar on the bacterial endotoxin test using rFC, explaining its current status as an alternative to the bacterial endotoxin test using the amoebocyte lysate.[7]

General chapter 2.6.14. *Bacterial endotoxins* gives a choice of six methods, A to F (gel-clot method: limit test, gel-clot method: quantitative test, turbidimetric kinetic method, chromogenic kinetic method, chromogenic end-point method, or turbidimetric end-point method), the Ph. Eur.'s aim would be to add a seventh method, method G, that could be used instead of any of the other methods. However, because the chapter has undergone International Harmonisation within the Pharmacopeial Discussion Group (PDG), no changes can be made to the chapter without the agreement of the other participating pharmacopoeias (United States Pharmacopeia and the Japanese Pharmacopoeia).[8] The topic is currently under discussion within the PDG.

Animal Welfare

The question of animal welfare is often raised in the context of rFC. The Ph. Eur. approach to this issue is laid out in its Introduction: *"**Use of animals**. In accordance with the European Convention on the protection of animals used for experimental and other scientific purposes (1986), the Commission is committed to the reduction of animal usage wherever possible in pharmacopeial testing, and encourages those associated with its work to seek alternative procedures. An animal test is included in a monograph only if it has clearly been demonstrated that it is necessary to achieve satisfactory control for pharmacopeial purposes."* Strictly speaking, rFC does not fall within the scope of the above-mentioned Council of Europe Convention, as the horseshoe crab is not directly used in pharmacopoeia testing. Nonetheless and very importantly, rFC avoids the use of a reagent extracted from a natural source and endangered species. As a single molecular entity, it also has higher standardization potential and as such represents significant technological progress. Last but not least, there is the crucial question of supply of the reagent: with horseshoe crabs absent from its coastlines, for Europe, the use of a recombinant alternative avoids potential supply shortages and a dependency on non-European countries; the potential supply concerns prompted by complete reliance on a single natural resource (the horseshoe crab) must also be taken into account. The recombinant source is an obvious step towards independence in this regard.

The pyrogenicity project fits perfectly within the scope of the "Replacement" aspect of the 3Rs, i.e. "technologies or approaches which directly replace or avoid the use of animals in experiments where they would otherwise have been used." Although the replacement of animals is a significant achievement in itself, there will be additional benefits from changing from *in vivo* to *in vitro* tests, including increased scope for standardization and reduced variability which, together, constitute a significant technological advancement. The situation will be reviewed in five years, after the respective texts have undergone their revision process.

Conclusion

Over the last 50 years the Ph. Eur. has addressed the question of pyrogenicity using the analytical techniques available at the time, moving from animal tests towards *in vitro* methods and therefore promoting the use of standardized methods for a better control of medicines in Europe. The Ph. Eur. has recently engaged on a path that will put an end to the use of rabbits in pyrogen testing and increase the use of synthetic reagents for the detection of bacterial endotoxins.[4]

Acknowledgements

The author wishes to thank the experts of the Ph. Eur., in particular the members of the Bacterial Endotoxin Test (BET) Working Party under the dedicated and enthusiastic leadership of Dr. Ingo Spreitzer, for their invaluable work.

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- **We continue to look for more guidance from the regulatory agencies:**
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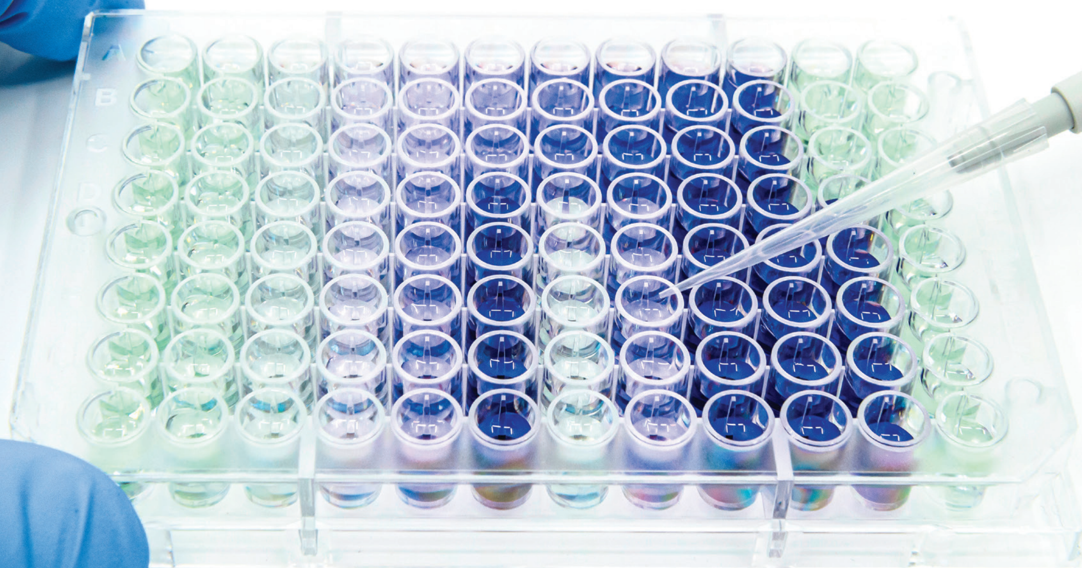
  - *How to evaluate the comparability data sets?*
  - *How many samples are needed to declare comparability of rCR to LAL?*



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The Truth of Endotoxin Values

Points for Consideration During Investigation of Aberrant BET Results

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Introduction

Bacterial Endotoxin Testing (BET) is mandatory for detection of Endotoxin/ Lipopolysaccharides (LPS) in parenteral drug products. This is important as LPS is one of the most potent stimulants of the human innate immune system. Over the last decades the Limulus Amebocyte Lysate (LAL) test has been established as the gold standard for the detection of LPS. In order to qualify a test sample, the endotoxin activity is determined based on a standard curve using reference or control standard endotoxins. BET is described in international pharmacopoeias (e.g., Ph.Eur, JP, USP). Thus, the test does not need to be validated per se, however product specific verification with tests for interfering factors is required.

In routine, most samples are analyzed in duplicate using one verified dilution of the product. In order to exclude test interference (i.e., inhibition/enhancement of the enzymatic reaction) positive product controls (PPC) are performed in addition. When all system suitability tests fulfill provided acceptance criteria a result is considered to be valid. In case not all acceptance criteria are fulfilled or unexpected results are obtained, further investigations are needed. Sometimes, these aberrant BET results are hard to understand.

Within a quality-controlled BET laboratory, operator and laboratory errors are often investigated relatively fast. However, there are additional challenging aspects which can influence a test result. In order to better understand inconclusive BET results the following points are often discussed:

- Variation in LAL reagents
- Variation in standard curve
- Representativeness of standard endotoxins
- Activity of endotoxin
- Alteration of detectable endotoxin
- Effects of (1→3)-ß-D-glucans

Is a Difference in LAL Reagent Results Possible?

Yes. The LAL reagents are derived from horseshoe crabs and are therefore of biological origin. It has been described that the lysate is a relatively crude mixture and is not a single purified enzyme. This means that the enzyme activity cannot be determined exactly for each lot of lysate manufactured. Furthermore, the manufacturing process includes the addition of buffers and detergents which contribute a further source of variability.[1] A reduced variability can be achieved by using recombinant Factor C reagents.

Can a Variation in the Standard Curve Effect the Test Result?

Yes. To quantify bacterial endotoxin, a standard curve is prepared in order to determine the endotoxin activity of a sample. Therefore, the quality of the standard curve is the basis of quantification. Using a linear standard curve, a change of only 1% in y-intercept can result in a change of up to 35% in measured endotoxin activity.[1]

In Figure 1, Y-intercept (Y-Achsenabschnitt) as a function of number of analysis (Analysennummer) from trending analysis is shown. All data points (full diamonds) fulfill the standard acceptance criteria. However, the typical observed variations may lead to increased/decreased test results depending on the y-intercept as small variations can lead to relative high variations in measured EU/mL.

Is Reference Standard Endotoxin Still Representative for BET?

Yes. Reference Standard Endotoxin (RSE) is the benchmark and allows comparability of test methods. Due to the heterogeneity of endotoxin, standardization of bacterial endotoxin tests was very challenging in the early time of BET. Only the introduction of RSE was the key factor to control the quality of BET, since *Limulus*-based approaches are ultimately biological assays, the lysates are intrinsically variable.[2] Moreover, recent challenges like LER and the implementation of recombinant tests brought up again discussions about Naturally Occurring Endotoxins (NOE). Advocates of NOE in the field of LER are refusing NOE when it comes to the comparison of test methods. It has been stated that NOE more closely mimics a real life contamination event,[3] but on the other hand it has been communicated that NOEs grown in laboratory are not representative of what occurs in nature. This contradictoriness clearly reflects the incongruous application of undefined endotoxin spikes during testing.

Can a Sample Composition Alter the Detectability of Endotoxin?

Yes. There have been a lot of publications about Low Endotoxin Recovery (LER) and endotoxin masking which can lead to underestimation of endotoxin contents.[2,4–6] Due to the presence of certain excipients or active pharmaceutical ingredients or combinations thereof, endotoxin can be masked. An example for the detectability of endotoxin in a typical LER matrix is given in Table 1. Thereby the detectability decreases although the endotoxin is not degraded and potentially hazardous.

Table 1. Detection of endotoxin over time in a typical LER matrix

Low Endotoxin Recovery Study	[EU/mL]
M1 Time Point 0 days	64.0
M2 Time Point 1 days	27.8
M3 Time Point 2 days	17.6
M4 Time Point 3 days	7.3
M5 Time Point 7 days	4.8

The data is sourced from Low Endotoxin Recovery - Masking of Naturally Occurring Endotoxin[6]

In order to reveal these effects so called LER studies are mandatory.

Therefore, undiluted samples are spiked with endotoxin and held for a certain period of time. More guidance for LER including strategies for demasking is found in the Technical Report No. 82 from PDA.

Do (1→3)-ß-D-Glucans Affect the Endotoxin Test Result?

Yes. The LAL test includes per se the Factor G reaction pathway which is described to react with (1→3)-ß-D-glucans.[7] This reaction pathway

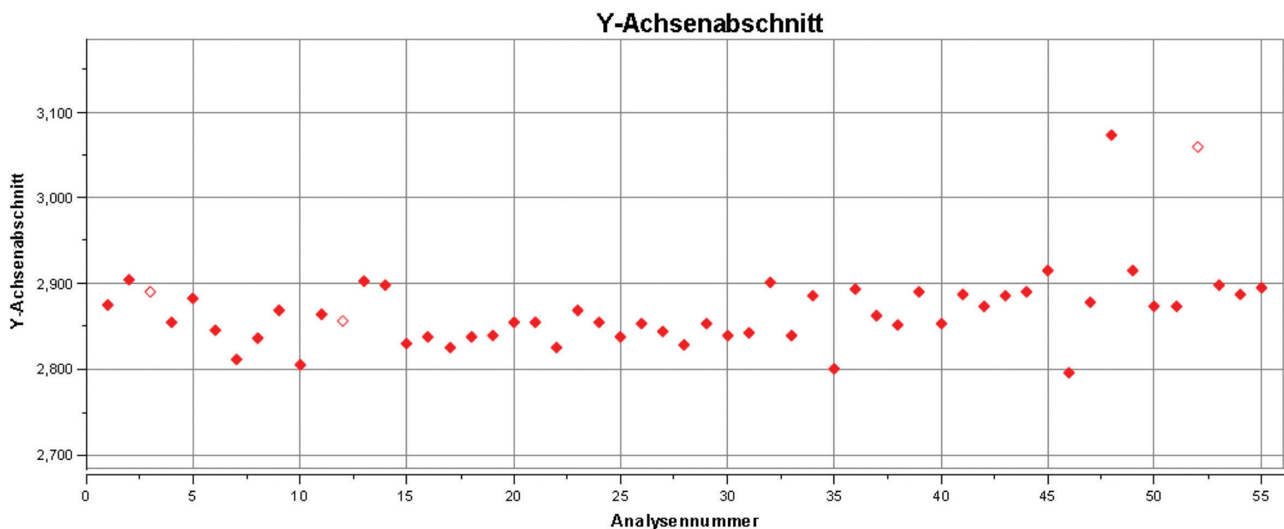

Figure 1. Y-intercept of standard curves from trending analysis

has been identified years ago. Interestingly this pathway is unequally pronounced in different LAL tests. There are also agents available to repress Factor G reaction pathway. Unfortunately, it is neither proven that a) all glucans nor b) that their full activity is blocked. Obviously, glucans are very heterogeneous and present in various aggregation states and can be derived from a variety of sources. Once present in a sample the absolute differentiation between LPS and glucans with LAL is virtually impossible.

In the following example, a routine in-process control sample during drug manufacturing resulted in an unexpected endotoxin test result. With a routine chromogenic LAL test method, 6.5 EU/mL (Table 2, arithmetic mean) was determined. With a turbidimetric LAL test method, 0.8 EU/mL (Table 3, arithmetic mean) was determined. The two test methods obtained valid results but with variations greater than the well-established 50% to 200%. Further analysis of (1→3)-ß-D-glucans revealed that the sample was contaminated by glucans (Table 4) which are most likely the root cause of the inconsistent results.

In order to determine the activity of endotoxin, only a recombinant reagent will allow determination of endotoxin, because of the lack of a Factor G reaction pathway.

Table 2. Analysis of in-process control sample with chromogenic LAL test

Dilution	Measured value [EU/mL]	Endotoxin content [EU/mL]	PPC [%]	Status
1:5	1.470	7.35	143	Valid
1:10	0.675	6.75	118	Valid
1:20	0.271	5.42	175	Valid

Table 3. Analysis of in-process control sample with turbidimetric LAL test

Dilution	Measured value [EU/mL]	Endotoxin content [EU/mL]	PPC [%]	Status
1:5	0.141	0.705	176	Valid
1:10	0.098	0.980	147	Valid
1:20	0.0372	0.744	149	Valid

Table 4. Analysis of in-process control sample with (1→3)-ß-D-glucans test

Dilution	Measured value [pg/mL]	Glucan content [pg/mL]	PPC [%]	Status
1:50	59.799	2990.0	97	Valid
1:100	34.952	3495.2	74	Valid
1:500	5.922	2961.0	89	Valid

Do Measured Activities Allow an Absolute Quantification of Endotoxin?

No. These test methods do not measure the amount of endotoxin/LPS, these tests rather measure activity (Endotoxin Units (EU)). The measurements quantify endotoxin activity which may vary from endotoxin to endotoxin. In the example below, supernatants of bacterial suspensions were analyzed using chromogenic LAL, recombinant reagent rFC and PBMC/IL6-based Monocyte Activation Test (MAT) (Table 5). While all tests were valid (i.e., according to European Pharmacopoeia 2.6.14, 2.6.30, 2.6.32) and manufacturer instructions, LAL and rFC tests resulted in the same order of magnitude, MAT measured values approximately 100 times less in one sample. Although the test results substantially deviate in MAT, this result should not be judged as incorrect. MAT is based on the reactivity of human monocytes (e.g., Toll-like Receptor 4) and LAL/rFC is based on the reactivity of Horseshoe Crab Factor C. Considering this fact, the result from MAT seems to be more relevant regarding the proximity/relevance of the test method to a patient.

Table 5. Analysis of supernatants of bacterial suspension using LAL, rFC and MAT

Sample	LAL [EU/mL]	rFC [EU/mL]	MAT [EU/mL]
Agrogbacterium radiobacter	207,000	242,500	2,000
Burkholderia multivorans	21,000	17,513	10,228

Conclusion

With respect to the examples provided it is difficult to rely on a single value. One single test method might not give the ultimate result. Although these tests methods have been used for decades, this does not imply that they can be used without considering their inherent advantages and disadvantages. Bacterial Endotoxin Tests are biological test systems and require careful interpretation as the relative detectability can vary more than the typical 50% to 200%.

Furthermore, application of the Monocyte Activation Test can be beneficial in providing more dedicated insights regarding the pyrogenic effects of a contamination. Generally, the test methods are only models to recapitulate the human situation. Despite all the challenges, available tests including recombinant tests are fast and sensitive methods to detect minute amounts of endotoxin.

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