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Forging the Path to Sustainable Pyrogen Testing: Insights from the Industry

News and views from the
6th Global Endotoxin Testing Summit

About the Global Endotoxin Testing Summit:

The Lonza Global Endotoxin Summit brings together testing experts, researchers, manufacturers, regulators, and conservationists to discuss critical trends and future directions for pyrogen testing in the industry's quality control (QC) sector. Despite the challenges of the COVID-19 pandemic, the September 2021 Summit went ahead successfully, held in a virtual format for the first time, and covered three time zones, with all recordings available on-demand. Sustainability, automation, and Lab 4.0 were the key themes presented by 24 expert speakers, attracting more than 1,000 attendees from over 70 countries. With the market for injectable pharmaceuticals expanding at an unprecedented pace and with greater focus being placed on the industry's environmental footprint, it's no surprise that one of the key themes of discussion at the 6th Global Endotoxin Summit was novel and sustainable pyrogen detection methods.

Pyrogens, or fever-inducing agents, are ubiquitous in the environment. Testing for their presence in parenteral pharmaceuticals and implantable medical devices is of

critical importance for patient safety, as these contaminants have the potential to cause major health issues, even at low levels, and can be introduced through raw materials or the production process itself. However, the majority of pyrogen testing assays used today still rely on experimental animals or animal components, such as the blood of the horseshoe crab, whose populations are in decline in certain parts of the world. Adoption of sustainable solutions is thus urgently needed.

In this article, we explore the broad range of insights shared from multiple perspectives at the 2021 Summit, covering the latest developments in sustainable pyrogen testing methods and, most importantly, practical hints on how to implement these new technologies in compliance with regulatory requirements.

From Rabbits to LAL — an Early Step Towards Sustainability

In his keynote speech, Dr. Jack Levin, professor at the University of California School of Medicine, San Francisco, and renowned endotoxin testing expert, reminded the audience that the first-ever pyrogen test, the rabbit pyrogen test (RPT), has been in use for more than a century. For the RPT, product samples are injected intravenously into rabbits, and changes in body temperature are monitored, with increases in temperature indicating pyrogen contamination.

With similar thresholds in pyrogenic response to humans, as well as simple injection and monitoring procedures, using rabbits provided a viable method for pyrogen detection at the time, and many human lives were saved. However, the RPT is slow and complex, with a lead time up to 55 days.

Levin went on to explain how the Limulus Amebocyte Lysate (LAL)-based test was developed in his lab in the late 1960s for the detection of bacterial endotoxins, the most prevalent of the pyrogens, and those most likely to contaminate drug products. The new test would ultimately go on to replace the RPT for bacterial endotoxin testing (BET). The LAL-based BET uses a lysate produced from amebocytes carefully collected from the horseshoe crab (HSC), a living fossil from the Paleozoic era, where coagulation cascade proteins initiate clotting in the presence of endotoxins.

"LAL is approximately a million times more sensitive than the response of humans towards bacterial endotoxins, meaning that the LAL test gives an enormous safety margin," Levin stated. To this day, the LAL test is regarded as the gold standard in BET, providing a simple and time-efficient testing method capable of sensitive detection of bacterial endotoxins.

Importantly, in addition to greater sensitivity and speed, the LAL test significantly reduced the number of experimental animals needed to conduct pyrogen testing, marking the first leap towards more ethical QC testing practices.

We warmly thank the following contributors to sessions

Americas

Dr. Jack Levin

Glenn Gauvry, Ecological Research & Development Group

Dr. Djikolngar Maouyo, PyroDex LLC, Inc.

Karen Zink McCullough, MMI Associates, LLC

Europe

Dr. Ingo Spreitzer, Paul Ehrlich Institute, Germany

Carmen Marin Delgado de Robles, Roche, Switzerland

Dr. Viviane Grunert de Fonseca, Roche, Switzerland

Dr. Eelo Gitz, Sanquin Reagents B.V, The Netherlands

Dr. Miriam Guest, Astra Zeneca, United Kingdom

Asia

Dr. Xiao-yong Xie, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences

Tan Jin (Lester) Xiang, Singapore Nature Society

Dr. Ming Che Yang, IUCN Horseshoe Crab Group, Taiwan

Protecting a Critical Natural Resource on a Global Scale

Although HSCs have been helping the biomedical industry safeguard patient health for more than 50 years, they are still a limited natural resource. Slow growth and late sexual maturity make HSC populations vulnerable to environmental changes and overfishing. Hence, incredible efforts have been made to protect these creatures in recent decades. In some areas of the globe, more work still needs to be done.

Mr. Glenn Gauvry, founder and president of the Ecological Research & Development Group, provided an update on the protection status of HSCs. Thankfully, in the US, HSC populations are rather stable due to well-established and successful sustainability programs. Careful harvest management, including the "bleed and release process", which involves safely releasing HSCs back into the sea within 36 hours after blood collection, has had a significant impact, reducing the mortality associated with LAL production to about 5 – 15%

In Asia, the situation is more difficult. HSC populations are in decline, as harvesting is largely unregulated, and best practices are often not adhered to. Panelists from several countries, Dr. Xiao-Yong Xie of the South China Sea Fisheries Research Institute, Dr. Ming Che Yang, International Union for Conservation of Nature (IUCN) in Taiwan, and Mr. Lester Tan of the Nature Society Singapore, introduced a variety of conservation initiatives that have been implemented to help stop illegal fishing and protect habitats, including community education programs to prevent the use of HSCs in traditional medicine and consumption of HSCs as a delicacy.

Dr. Yang also presented scientific approaches that are being used to analyze genetic diversity and develop programs for artificial breeding, growth, and release, all of which are helping to monitor and further improve conservation efforts.

The Drive for Sustainable Alternatives to Horseshoe Crab Blood

Despite conservation efforts, global population growth, medical care advancements, and expanding regulatory requirements are driving an anticipated 7% compound annual growth rate for the LAL market, putting additional pressure on HSC populations and highlighting the need to urgently consider sustainable solutions. A critical development in the biomedical industry's continued commitment to relieve the burden on HSCs comes in the form of the recombinant Factor C (rFC) assay.

The rFC test was developed and commercialized first by Lonza in 2003 to provide a sustainable alternative to LAL while helping to meet growing demand. Instead of the entire set of HSC blood proteins, the assay uses a recombinant form of Factor C, the first protein of the coagulation cascade that directly reacts with endotoxins. The potential impact of such a solution is hard to understate.

As Gauvry pointed out: "A 30 L production run of rFC can produce the equivalent to bleeding 6,000 horseshoe crabs."

2018 marked a significant milestone for the rFC assay, when it was used for testing of Emgality®, the first FDA-approved drug to use this method for its release. In the same year, a second vendor introduced a commercial rFC test, increasing the reliability of supply and helping to facilitate regulatory acceptance. Several studies demonstrated the feasibility and specificity of rFC tests, as well as comparability to LAL methods, leading to their recognition as alternative pharmacopeia assays in the European Pharmacopoeia (Ph. Eur.) in 2021. In the US, rFC tests are still regarded as alternative non-pharmacopeia assays, requiring a method validation.

At the Summit, Miriam Guest, Associate Principal Scientist leading Astra Zeneca's "21st Century Microbiology Technology Strategy" welcomed the concept of reducing the use of LAL reagents. "We seek to balance patient protection with the responsible management of shared natural resources and coastal biodiversity and believe all sustainability-enhancing options should be evaluated," she explained.

With effective, sustainable LAL alternatives available, the next step to achieving global adoption and implementation is the creation of a diligent validation process. Significant progress is already being made here.

Carmen Marín Delgado de Robles and Viviane Grunert da Fonseca presented Roche's rFC validation strategy adhering to the 3Rs principle ("Reduce, Replace, Refine"), enhancing animal welfare while ensuring patient safety. The two-step validation approach, built on risk assessment and statistical evaluation of non-inferiority testing of the rFC and LAL assays, was discussed, serving as an example for other companies looking to implement rFC into their BET programs.

The availability of automated rFC tests, a growing number of use cases, and increased company and individual efforts to meet sustainability objectives will continue to drive the adoption and smoother validation of rFC tests in the near future.

Goodbye to Rabbit Pyrogen Tests for Good: Closing the Gap for Non-Endotoxin Pyrogens

Whereas most of the discussions focused on endotoxin testing, Dr. Djikolngar Maouyo, PyroDex, brought non-endotoxin pyrogens into the spotlight. "Compared to bacterial endotoxins, the molecular structures of non-endotoxin pyrogenic contaminants are very diverse, originating from sources such as Gram-positive bacteria, mycoplasma, fungi, viruses, chemicals, and other impurities. Many clinical trials fail not because of drug inefficacy or device engineering flaws, but because of contaminants that are undetected by current safety methods," he noted.

While the RPT detects both endotoxin and non-endotoxin pyrogens, LAL or rFC assays only detect endotoxin

pyrogens. In Europe especially, implementation of *in vitro* assays are needed as the RPT chapter will be discontinued in Ph. Eur. in 2026, a significant development demonstrating the deep commitment of both the regulatory authorities and the industry to animal welfare.

The Monocyte Activation Test (MAT) offers a viable option. The MAT mimics the human immune reaction to pyrogens, where monocytes respond to pyrogenic contaminants with the production of inflammatory cytokines. Samples are incubated with a monocytic cell source (e.g., peripheral blood mononuclear cells (PBMCs)), culture medium, and an optimized culture medium supplement delivering proteins supporting the recognition of pyrogens. After incubation, levels of interleukin-6, a cytokine demonstrated to correlate with increases in body temperature, are determined. While in the US Pharmacopeia (USP), MAT is mentioned as an alternative method (USP <1085>), it was already implemented in Ph. Eur. in 2009.

Dr. Eelo Gitz, Sanquin Reagents, described how to broaden

Identification		MAT	RPT	BET
Pyrogens	Endotoxin	✓	✓	✓
	Other bacteria	✓	✓	
	Yeast / Fungi	✓	✓	
	Virus , DNA, RNA	✓	✓	
	Particles	✓		
Limitations	Lipids	✓	✓	
	Proteins (Bioprocess)	✓		✓
	Blood therapeutics	✓		✓
	Cellular therapeutics	✓		✓
	Immunogenic biologics	✓		✓
	Vaccines	✓	✓	✓
Experimental controls		✓		✓
Pyrogenicity		Human	Mammal	Endotoxin
Experimental animals		No	Yes	No*

The MAT is best suited to detect pyrogens in biologics.
*Utilize horseshoe crab blood (natural resource)

Figure 1. Overview of tests detecting endotoxin and non-endotoxin pyrogens.

access to this promising test: "Instead of fresh cells, we manufacture cryopreserved PBMCs, which enable cells to be more easily stored and shipped, and allow the production and extensive qualification of larger batches with consistent quality."

Gitz also presented data on a novel MAT variant developed in his lab. It was found that culturing PBMCs in human serum, instead of the historically used fetal calf serum, led to improved reactivity towards non-endotoxin pyrogens and lower interference with human blood products. Based on these results, a commercial MAT kit set containing human serum was developed and will be available through Lonza in 2022.

Despite these innovations and the impending changes to Ph. Eur., Dr. Ingo Spreitzer, deputy head of Section 1/3 Microbiological Safety of the Paul-Ehrlich Institute and chair of EDQM Working Party "Bacterial Endotoxin Test," Germany, noted that RPTs are still widely used. Although the MAT is a 'complete' alternative pyrogen test and provides higher sensitivity compared to RPT for bacterial endotoxins and non-endotoxin contaminants, it is more time-consuming with lower throughput than LAL tests.

To eliminate RPTs entirely, Spreitzer suggested combining the advantages of both alternative BET and MAT analyses. "We recommend applying BET throughout the production process and adding MAT for the final product release. This way, we see the full picture, including synergisms between endotoxin and non-endotoxin pyrogens," he stated.

Towards Sustainability in Pyrogen Testing

Testing for both endotoxin and non-endotoxin pyrogens ensures the safety of parenteral products and implantable medical devices, increases the success rate of clinical trials, and is vital for overall patient safety. It's clear that, over time, novel recombinant and cell-based assays are expected to take over, both increasing sustainability and animal welfare while also addressing the industry's growing demands for reliable supply.

Access more on the latest sustainability developments in pyrogen testing, including MAT and rFC tests.

Stay Tuned for More Highlights from the 2021 Global Endotoxin Testing Summit

In our next article, we explore another key theme from the Summit — the importance of digitalization and lab automation for sustainable, future-proofed QC operations, and provide a glimpse into the opportunities of QC-Lab 4.0.

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