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From LAL to rFC Assays

A More Sustainable Future for Endotoxin Testing

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Bacterial endotoxins are pyrogenic lipopolysaccharides found in the outer cell membrane of Gram-negative bacteria. Ubiquitous in the environment, endotoxins can easily enter the patient's bloodstream accidentally via contaminated parenteral drugs or implantable medical tools such as intravenous infusion devices.

From LAL to rFC Assays

An introduction to bacterial endotoxin testing

An immune response can result in debilitating symptoms such as fever and vomiting, and can even be fatal. As such, bacterial endotoxin testing (BET) is an essential safety requirement in the pharmaceutical and biomedical industries.

The Tachypleus and Limulus Amebocyte Lysate (TAL/LAL) assays are currently the most commonly used tests for detecting bacterial endotoxins. These assays are produced using whole cell lysates of specialized immune cells (amebocytes) found in the blood of various species of horseshoe crabs. Specifically, the LAL assay uses amebocytes from the *Limulus polyphemus* species, which inhabits eastern coastal areas of North America, whereas the TAL assay uses amebocytes from the *Tachypleus gigas* and *T. tridentatus* species found in various regions in Asia.

Amebocytes form part of the horseshoe crab's innate immune defense system and their granules naturally form a clot when they encounter endotoxins, providing a powerful reagent for detecting clinically harmful levels. Indeed, after its introduction over 50 years ago[1], the LAL assay has become universally regarded as a highly sensitive and specific method that reliably detects endotoxin contamination. The applications of LAL are also extensive. For example, it has been used to detect endotoxins in pharmaceutical preparations, on-site tests of short-lived radioisotopes, food quality testing, and environmental monitoring.

The importance of horseshoe crab conservation

Despite the value of the LAL assay, there are growing concerns about the effects of the LAL/TAL industry on global populations of horseshoe crabs. These "living fossils" represent a remarkable feat of evolutionary resilience, having survived on Earth for an astounding 450 million years. They also play a vital role in the wider ecosystem. For example, in the Delaware Bay Estuary on the eastern coast of the United States, tens of thousands of shorebirds, such as the red knot (*Calidris canutus*), rely on surplus Atlantic horseshoe crab eggs for critical energy reserves to make long migratory flights.

Atlantic horseshoe crabs have long been harvested as bait for commercial American eel and conch fisheries. Significant efforts have been made to prevent these activities from having a detrimental impact on the Atlantic horseshoe crab populations, and to protect the supply of LAL. For example, since 1998, interstate harvesting quotas have been enforced by the Atlantic States Marine Fisheries Commission (ASMFC). Moreover, the non-profit Ecological Research and Development Group (ERDG) has developed a variety of horseshoe crab conservation initiatives such as the introduction of bait bags to reduce the number of crabs used as bait, in

addition to the "Just flip 'em!™" Program, an initiative that encourages beachgoers to "flip" stranded horseshoe crabs during the spawning season. In 2019, the stock status of Atlantic horseshoe crabs was found to be either neutral or good in most eastern coastal areas of the USA.[2]

However, the situation is different in Asia, where no national regulations on harvesting or bleeding practices exist. Although some regional regulations are in place (such as along the coast of the southern region of Guangxi in China), their enforcement across typically huge geographical areas is extremely challenging. Consequently, two of the three Asian horseshoe crab species are routinely bled to death in the production of TAL and then sold for human consumption. Along with unregulated commercial fishing, these unsustainable practices are causing population decline in some areas. For example, in China, horseshoe crabs were once commonly found from the mouth of the Yangtze to the Guangxi coast. But none have been seen along the coast of Zhejiang province for over a decade, and there are now only occasional sightings in Fujian, Guangdong and Guangxi.[3]

The drive towards a different endotoxin testing approach

The pharmaceutical and biomedical industry's continued reliance on horseshoe crabs for BET could be problematic for several reasons. As TAL becomes progressively limited through the decline of the Asian horseshoe crabs, this may place too great a burden on the North American populations and the LAL industry for it to be sustainable in the future. This problem is exacerbated by the growing demand for LAL/TAL exhibited by the vaccine development and production industry, such as that seen in the burgeoning Asia-Pacific market.

Additionally, the emergence of personalized medicine, such as cell or gene therapies, will mean each product will need to be tested individually, requiring more frequent endotoxin testing and subsequently placing even greater demand on lysate-based tests. As such, the industry has long recognized the need to change its approach and widely adopt more sustainable, animal-free endotoxin testing methods that do not affect horseshoe crabs or the wider ecosystem in which they play a key role.

Although such change can take time and require significant investment in the short term, these initial costs can be far surpassed by the long-term benefits. One illustrative example is the replacement of the traditional qualitative Rabbit Pyrogen Test (RPT) by the LAL assay for many pharmaceutical and biomedical products. This change ultimately benefited the industry by providing an ethically more acceptable assay with higher sensitivity that can be run with relative ease and speed. Moreover, the LAL assay enables quantitative measurements of endotoxin to demonstrate the allowable limit for license specifications (for example, large volume parenterals must not contain greater than 5 IU endotoxin per kg body mass).[4] This demonstrates how widespread change in the industry's approach to endotoxin testing is not only achievable but can also be extremely valuable.

The sustainable recombinant Factor C (rFC) assay

One sustainable, animal-free endotoxin testing method is the recombinant Factor C (rFC) assay. This is the synthetic version of Factor C, an element found in the amebocyte cells of horseshoe crabs. Both the natural and synthetic versions of Factor C activate the clotting cascade when triggered by endotoxins (Figure 1) to detect endotoxin presence and provide a measurement of the amount.

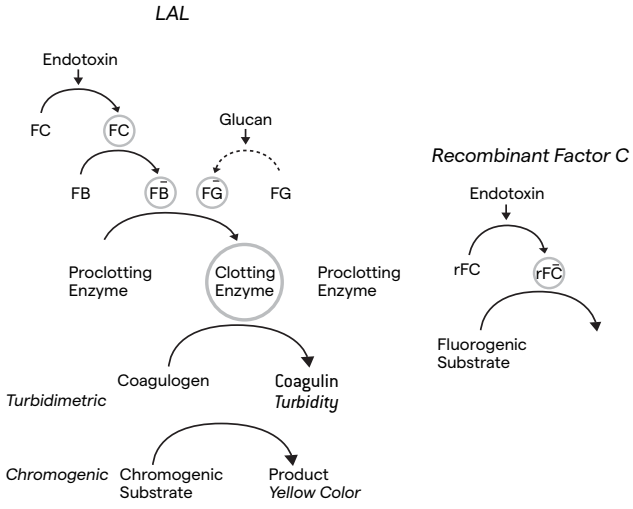

Figure 1.
Natural Factor C initiates a clotting cascade measured using turbidimetric and chromogenic assays. Lonza's synthetic PyroGene® Recombinant Factor C Assay triggers a similar reaction, but the output is measured using a simpler fluorogenic assay that does not rely on an amplification cascade.

Lonza first recognized the need for a sustainable animal-free endotoxin testing method 15 years ago, when the company had the forethought to start developing a synthetic, recombinant solution: the PyroGene® Recombinant Factor C Assay. This is an end-point fluorescence test that is biochemically and practically equivalent to LAL, which uses rFC to cleave a fluorogenic substrate and subsequently reveal a measurable fluorescent signal (Figure 2).

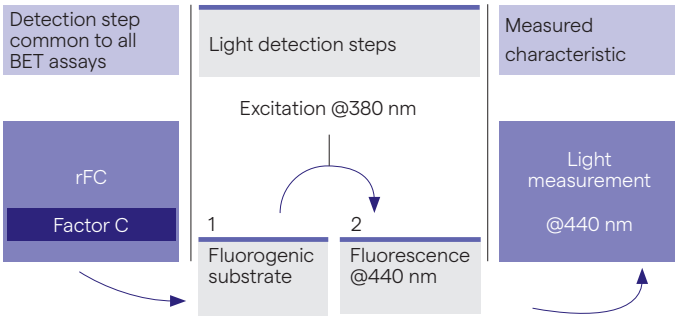

Figure 2.
The PyroGene® Recombinant Factor C Assay works through the same initial binding step as LAL BET assays, except that it only requires the binding of one protein to produce a measurable fluorescent signal.

Endorsed by the 2012 FDA guideline and the European Pharmacopoeia (Ph. Eur. 5.1.10), the rFC assay has been shown to perform comparably or even better than TAL/LAL-based approaches.[5] As well as improved assay performance, it also offers several other advantages. For example, it protects against TAL shortage and the potential impact of the growing demand for LAL. Also, as it is not reliant on animal resources, it helps meet ethical and regulatory initiatives to minimize animal use in testing (such as the 3Rs principle to Replace-Reduce-Refine).

Additionally, the synthetic PyroGene® Assay provides improved lot-to-lot consistency compared to the LAL test, which exhibits some variability between different batches of the same product when obtained from different crabs and at different collection times. Other key benefits of this synthetic method are that it is easy to use and offers enhanced endotoxin specificity (with a sensitivity range of 0.005 – 5 EU/mL or greater) and statistically more robust spike recovery.

Recent regulatory developments regarding recombinant Factor C

Despite the benefits of rFC, the assay has historically been listed as an "alternative method" in Pharmacopeial guidelines. This meant that its use in BET required additional validation steps, which deterred some manufacturers from using it. However, in September 2018, Eli Lilly's new migraine prevention drug, Emgality® (galcanezumab), was the first drug approved by the US FDA that uses the rFC assay instead of traditional LAL-based methods for endotoxin detection in clearance tests.[6]

Following this, in January 2019, the European Pharmacopoeia launched a public consultation on a new general chapter, 2.6.32. Test for bacterial endotoxins using recombinant Factor C (rFC)[7] and in September 2019, the United States Pharmacopeia (USP) announced plans to develop a draft chapter <85> Bacterial Endotoxins Test, which will include the addition of rFC assays. Subsequently, the Japanese Pharmacopeia published a general information draft of Chapter 4.01 discussing alternative bacterial endotoxin testing methods using recombinant protein-reagents.[8] Most recently, the Chinese Pharmacopeia has listed and described rFC as a new compendia method.[9] Overall, these recent changes suggest that rFC is becoming a more accepted method for bacterial endotoxin testing and could lead to its wider adoption in the pharmaceutical and biomedical industry.

The PyroGene® Recombinant Factor C Assay utilizes liquid reagents that eliminate the need for reconstitution and reduces waste of reagents.

A changing tide in bacterial endotoxin testing

There is clearly a growing recognition of the importance of a sustainable approach to bacterial endotoxin testing. Although there is every indication that the rFC assay is gaining momentum as an animal-free endotoxin test, the time and investment required for its widespread adoption means that it is unlikely to replace LAL-based tests completely for many years to come. Encouraging change is even more challenging given LAL manufacturers have a vested interest in the LAL-based approach.

The generation of further evidence-based and accurate proof-of-principle data is vital to continue the drive away from animal-derived lysate-based tests and adopt more sustainable testing practices. Indeed, as more companies like Eli Lilly take the lead and obtain regulatory approval for more drugs that use rFC in clearance tests, other organizations are likely to follow suit and gain the many benefits that a synthetic, recombinant approach offers.

Closing remarks

The pharmaceutical and biomedical industries cannot ignore the need for a sustainable approach to bacterial endotoxin testing. Amid growing concern for global horseshoe crab populations and their key role in the wider ecosystem, the need for an animal-free endotoxin test has become paramount. Also, with the TAL supply under threat and the demand for BET increasing globally, the LAL industry is unlikely to be able to sustain this huge burden in the future. The rFC assay has emerged as an animal-free technology that is comparable to LAL and offers significant benefits such as ease of use and minimal lot-to-lot variability. In the past, the rFC assay has struggled to be accepted as anything more than an 'alternative' test in various Pharmacopeia. However, with newly approved drugs like Emgality® using the rFC assay as a clearance test, and the growing recognition of its importance by various Pharmacopeia, widespread adoption of the rFC method could be on the horizon.

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