Advances in Using Sustainably Generated Amebocytes to Ensure Affordable and Effective Endotoxin Testing

Authors: D. Kilbank, W. Tutak, G. Kaufman

Abstract

The Limulus Amebocyte Lysate (LAL) assay, derived from horseshoe crab blood, has been the global gold standard for endotoxin detection for more than fifty years, yet its reliance on wild crab harvesting creates significant ecological, supply chain, and regulatory challenges. Recombinant alternatives (rFC, rCR, MAT) have emerged, but their limited functional equivalence and reduced sensitivity pose serious risks to patient safety and reliability. MygoGenesis, through its proprietary Mygotic Process™, has developed AmeboGenesis™ (AG), a platform for generating unlimited, bioidentical amebocytes from reprogrammed somatic cells. These laboratory-grown amebocytes replicate the natural immune complexity of horseshoe crabs while ensuring sustainable, consistent, and scalable production. The resulting aLAL™ lysate demonstrates improved reproducibility, broader sensitivity across endotoxin variants, and compatibility with standard testing formats, while alleviating ecological pressures on marine populations. In addition to transforming bacterial endotoxin testing, AG-derived peptides and components hold potential for novel therapeutic and diagnostic applications, including antimicrobial peptides, sepsis management, and regenerative medicine. This breakthrough establishes a path toward reliable, sustainable, and globally harmonized endotoxin detection—advancing patient safety, preserving biodiversity, and opening new frontiers in biopharmaceutical innovation.

Keywords: endotoxin detection, LAL, aLAL™, AmeboGenesis™, Mygotic Process™, sustainable biotechnology, recombinant limitations

Corresponding Author: Daniel Kilbank - daniel.kilbank@mygogenesis.com

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The Limulus Amebocyte Lysate's (LAL's) critical role in endotoxin detection

***A*uthors:** D. Kilbank, W. Tutak, G. Kaufman

The Limulus Amebocyte Lysate (LAL) test, developed in the 1970s and derived from the blood of horseshoe crabs (HSCs), has been the gold standard for detecting bacterial endotoxins (pyrogens, such as lipopolysaccharides-LPS from Gram-negative bacteria) ever since. Presence of endotoxins can cause severe pyrogenic reactions, including fever, septic shock, and potentially death. By leveraging the natural clotting reaction of HSC amebocytes, the LAL test offers highly sensitive and specific detection of endotoxins essential for safeguarding injectable pharmaceuticals, vaccines, biologics, and medical devices. Mandated by global regulatory authorities, including the U.S. FDA and other international agencies, with guidance from the United States Pharmacopeia and corresponding global pharmacopoeias (e.g., PMDA, EMA, etc.), The LAL test is implemented in gel-clot, turbidimetric, chromogenic, and fluorogenic formats.

The growing biomedical demand and commercial fishing reliance on harvesting wild HSCs have raised serious ecological and animal welfare concerns, necessitating a reevaluation of the existing endotoxin detection methods. This has led to the recent proposal and implementation of ethically responsible and scientifically viable alternatives, including recombinant Factor C (rFC) and recombinant cascade reagent (rCR) technologies. These recombinant methodologies are a suboptimal substitute for the natural cascade of proteins and immune responses produced by the HSC's immune system. They are limited to artificially generating proteins raised against highly specific bacterial strains, and the narrow, selective nature of this process significantly compromises performance and jeopardizes human health care. This facilitates regulatory harmonization and drives innovations in endotoxin detection to improve testing methods while preserving marine biodiversity and ensuring reliable and sustainable testing solutions. The global pyrogens testing market is expected to grow from USD 2.9 in 2024 to USD 3.7 billion by 2027. Traditional LAL testing accounts for 90 % of the market. Recombinant technologies, including rCR and rFC, make up 9%, primarily in Europe and the US, while monocyte activation test (MAT) and other *in vitro* methods account for < 1%.

Key challenges associated with traditional LAL endotoxin testing

Despite its widespread regulatory acceptance and critical role in endotoxin detection, the LAL testing method faces significant challenges related to scalability and sustainability. Its reliance on collected HSC blood raises ethical and ecological concerns, particularly due to climate change and environmental stressors leading to a global decline in crab populations, despite the enactment of new conservation programs and regulations. Over 1 million HSCs are harvested annually in North America, with post-bleeding mortality rates of at least 30% and observed sublethal impacts on their behavior and reproduction[1]. Crabs require 20 years to reach reproductive maturity, so disruptions to adult populations can have long-term impacts on global demographic stability. As a keystone species, their decline has known broad ecological

consequences (e.g., crabs and their eggs are preyed upon by fish, migratory birds and other predators)[2]. This fragile supply chain (reliant on seasonal collection in the wild) is increasingly vulnerable to the factors of climate change, habitat loss, use for commercial fishing, and regulatory pressures (some states banning the collection of HSCs), which can disrupt global biomanufacturing and increase costs and efficiency of LAL testing.

Furthermore, LAL production from wild HSCs introduces batch-to-batch variability of raw material due to differences in individual, geographic, and seasonal factors, as well as inconsistent processes for amebocyte extraction and lysate formulation between the licensed LAL manufactures. In addition, these individual crabs are subjected to constant and variable immune challenges, leading to fluctuations in their immune systems/responses and ultimately affecting the quality and consistency of the resulting bleed batch. These factors can contribute to false positive or negative results and challenges in detection reliability, standardization, and scalability of the LAL tests. Further, reduced sensitivity directly impairs the broad detection of harmful bacteria or fungi and compromises diagnostic reliability. The current LAL formulation steps, which utilize batch-pooled blood from multiple crabs, are modified and narrowly modulated against a defined set of endotoxin standards, thereby reducing the breadth and depth of natural detection limits to a narrow scope of pathogens. They increase the cost of medical treatments and endanger patient safety.

Supplier dependence on limited number of companies (e.g., LONZA, Charles River, ACC, Wako, etc.) raises concerns over cost and supply stability. Even the most advanced LAL harvesting methods still rely on wild populations, posing concerns about sustainability and consistency. Integrated solutions such as Low Endotoxin Recovery (LER) mitigation and automation introduce further complexity, costs, and training requirements that are particularly burdensome for small labs and increase cost of medical treatments.

Limitations of alternatives to LAL endotoxin testing (Recombinant Reagents)

Ethical concerns and growing pressure from conservation groups and regulatory bodies are prompting an erroneous and very dangerous shift toward synthetic alternatives to the LAL test which has been the gold standard for over 50 years. Main alternative methods to the LAL test include recombinant Factor C (rFC), recombinant cascade reagent (rCR), and monocyte activation test (MAT). These tests offer ethical and sustainable advantages by eliminating the need for animal-derived components, but this comes at the direct cost of a substantial reduction in their ability to detect a broad spectrum of endotoxins at lower concentrations. Recombinant based tests (e.g., Trillium by Charles River), with fixed reagent ratios and limited number of functional enzymes, make them less effective to detect endotoxin in complex or inhibitory natural matrices. Recombinant tests are more sensitive to pH shifts, ionic strength, and interfering substances like chelators or detergents, increasing the risk of reporting false positive and negative results[3].

Recombinant assay platforms may show low endotoxin recovery (LER) in the presence of masking agents, unless demasking agents or naturally occurring endotoxin (NOE) spikes are used[4,5]. In addition, it is estimated that the false negative rate for recombinants is as high as 30%, increasing concerns for product safety and higher patient mortality. Recombinant assays suffer from a lack of global regulatory harmonization, reduced sensitivity to NOE variants, and

reliance on specialized equipment and reagents that are not standardized or widely accessible. Constant reformulation and efforts to create recombinant solutions that mirror the natural complexity and evolving landscape of living bacteria constitute an exceptionally complex task. Ongoing innovation and improvements will be essential to maintain the alternative recombinant tests' sensitivity and regulatory compliance while enhancing sustainability, reproducibility, and reliability.

Additional concerns include rFC and rCR's inability to detect (1,3)-β-D-glucans, which is important for identifying fungal contaminants, and variable performance in the presence of certain biologics or excipients[6,7]. The rFC and rCR tests also face biological hurdles, such as the need for accurate expressions and post-translational modifications of proteins/components that naturally interact in tightly regulated pathways within native HSC amebocytes. Producing functionally active, properly folded, and stable recombinant proteins in heterologous systems remains technically demanding. Recombinant cascade platforms (e.g., Trillium) may show reduced LER after hold times (the period during which the spiked sample remains stored prior to the assessment of endotoxin recovery) with reference standards endotoxin (RSE) or control standard endotoxin (CSE) spikes. Their use requires spike recovery testing with NOE (derived from whole cell wall extract of Gram-negative bacteria) and RSE, CSE lot variability assessments, temperature-controlled hold-time, and documentation of matrix-specific performance[8,9].

Alternative Monocyte-based test methods (MAT), while biologically relevant, present challenges including human donor variability, ethical sourcing concerns, technical complexity, and inconsistent outcomes due to cross-reactivity and matrix interference.

While the U.S. and other Pharmacopoeias continue to endorse LAL, the European Pharmacopoeia has adopted rFC and rCR tests, reflecting inconsistent and shifting global regulatory landscape. FDA allows to use the alternative methods to Bacterial Endotoxin Test (BET) after validating them in accordance with USP Chapter <1225> and <1226>[10,11]. Additional validation requirements increase overall expenses for the biomedical industry and force the multinational pharmaceutical companies to navigate conflicting standards and guidance documents. The U.S. Pharmacopoeia (USP) recognizes only LAL tests under chapter <85> often necessitating dual validation alongside alternative tests, which adds cost and time. Transitioning to rFC and rCR (under chapter <86>) involves revalidation, staff training, SOP revisions, and infrastructure upgrades, which can delay quality control workflows and deter adoption[12].

Overall, the promise of recombinant based technologies is overshadowed by concerns about patient safety since these tests have shown limited functional equivalence to native systems[12,13]. Alternative tests also face challenges such regulatory fragmentation, high investment barriers, infrastructure demands, and insufficient industry-wide coordination. Addressing these challenges will require unified efforts from regulators, test developers, and end-users.

Advanced strategies for overcoming challenges associated with traditional and alternative amebocyte-based endotoxin detection systems

The proprietary Mygotic Process™, developed in our laboratories, transforms somatic cells (originated from HSC muscle leg that can be regenerated) into autologous totipotent embryonic

cells. Under the proper conditions, these cells undergo unlimited divisions, banking, and differentiation into bio-identical AmeboGenesis (AG) amebocytes. The lab-generated cells are collected to obtain LAL and to prepare endotoxin test kits (Figs. 1 and 2). Continuous expansion of these amebocytes provides a consistent population of single-sourced cells and an unlimited supply of amebocytes. This offers a sustainable and reliable alternative to traditional Limulus Amebocyte Lysate production, which relies on harvesting blood from over 1 million wild HSCs per year. It supports ecological conservation by alleviating pressure on crab populations, enhances reagent consistency by reducing batch-to-batch variability, and ensures year-round availability of fresh LAL to meet rising global demand. This process enables the production of new amebocytes from HSCs multiple times per year, ensuring that the latest immune responses from marine regions are obtained and maintained. It also significantly enhances the immune response derived from amebocytes generated by individual crabs. Since the cells are cultured in a controlled bacteria- and fungal-free environment, the immune response is primed to detect any kind of pathogen introduced to the system. Continuous cell availability improves reproducibility, mitigates contamination risks, and promotes ethical and environmentally responsible practices in pharmaceutical, biotechnology, and medical device industries.

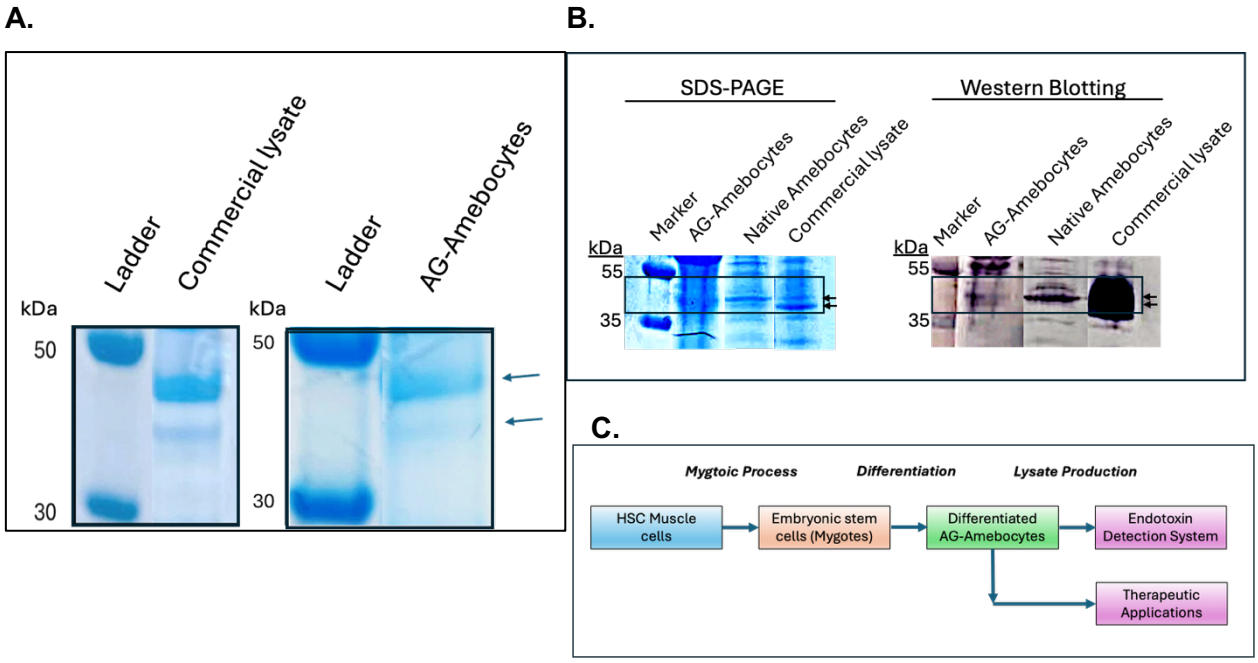
A stable and scalable supply of standardized, laboratory-grown amebocytes will also reduce regulatory friction by increasing confidence in the long-term viability and safety of LAL. An unlimited supply of high-quality amebocytes will eliminate the need for transition to recombinant alternatives, which face harmonization challenges, higher costs, and technical limitations. Stable and sustainable supply HSC derived lysates will reduce revalidation burdens, avoid infrastructure overhauls, provide a cost-effective testing solution for laboratories and, most importantly, enhance patient safety. Lysates obtained from the AmeboGenesis (AG) amebocytes exhibit greater consistency, enhancing endotoxin test reproducibility and sensitivity while minimizing the need for repeated validations and troubleshooting in product testing. Stable production of AG-Amebocytes will stabilize pricing, mitigate limited supplier risks, and enhance global supply chain resilience.

In addition, customizable lysate preparation allows for the adjustment of Factor C, Factor B, and pro-clotting enzyme levels for matrix-specific optimization. Fresh lysates can be buffered post-lysis to match sample conditions, improving recovery and reducing false negatives[14]. Native lysates demonstrate robust detection of various LPS conformations, including vesicle-bound and aggregated LPS[15], lowering the risk of LER and reducing the need for demasking agents. They are compatible with chromogenic, fluorogenic, and turbidimetric formats, offering flexible endpoint and kinetic control, which is critical for masked or slow-reacting endotoxins. Fresh lysates also avoid reconstitution artifacts and can be validated with NOE, demonstrating robust performance across different temperatures and storage conditions (e.g., reconstituted lysates may be stored at 2-8°C for up to one day, or at -20°C for up to four weeks). Native LAL lysate is considered a USP <85> compendial standard for endotoxin detection and it remains the globally accepted benchmark for product development and regulatory compliance.

Comparison of AG-Amebocyte LAL to traditional LAL from Native HSC blood and commercial lysate

Fig 1. **A**: Immunoprecipitation data comparing Factor C light chain expression in LAL commercial lysate versus AG-Amebocyte cells (developed through the Mygotic Process™). The arrows indicate two forms of factor C light chain: Top arrow-unprocessed; Bottom arrow – processed. **B**: SDS-PAGE (left) and Western blotting (right) analysis of factor C expression (black box) in AG-Amebocytes, native amebocytes, and commercial lysates. Arrows indicate unprocessed (top) and processed (bottom) protein bands. **C**: The development of AG-Amebocytes for LAL testing platform and therapeutic intervention.

Comparison of endotoxin detection and onset times by AG-amebocytes (AGA) to traditional detection systems.



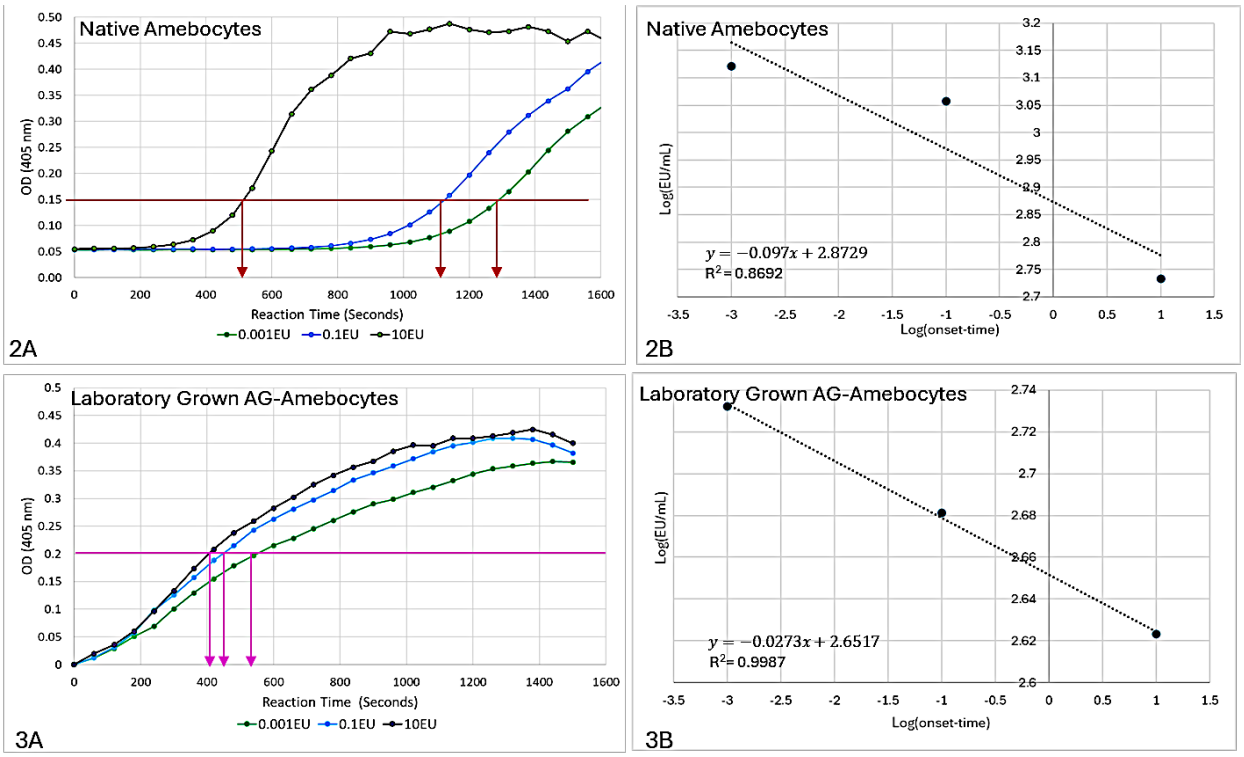


Fig. 2 A: Endotoxin dose-response kinetic curves generated from native amebocytes lysate (LAL) reacting to series of standard endotoxins. Typical sigmoidal shape of reaction can be observed for the tested native LAL. The red line shows the onset time, and the arrows indicate representative time points. The samples were processed at AG laboratory. **B**: Standard curve for LAL samples was plotted by graphing log(onset time) as a function of log(endotoxin concentration) using the three representative time points obtained in Fig. 2.A. Linear regression analysis calculated negative slope with a value of -0.097. Coefficient of determination (R^2) was calculated to be 0.8692.

Fig. 3 A: Endotoxin dose-response kinetics curves generated from AG-amebocytes (AGA) reacting to series of standard endotoxins. Graph of the reaction has exponential shape with rapid onset. The purple line shows the onset time, and the arrows indicate representative time points. The samples were processed at AG laboratory. **B:** Standard curves for AGA samples were plotted by graphing log(onset time) as a function of log(endotoxin concentration) using the three representative time points obtained in Fig. 3.A. Linear regression analysis indicated shallower negative slope of -0.0273. Coefficient of determination (R^2) for AGA was calculated to be 0.9987 and meeting current compendial requirements of r > 0.980.

Innovative therapeutic applications based on AG-Amebocyte components

Purification of innovative products from the laboratory grown amebocytes and hemolymph-derived components, such as LAL factors, Tachyplesin, Polyphemusin, Big defensin, and other peptides can lead to the development of multifunctional filtration systems applicable to water treatment and therapeutic applications. These products could support the prevention and

treatment of sepsis via blood filtration, serve as diagnostic tools for various infections, and potentially contribute to cancer therapies[16].

The peptides found within amebocyte cell granules may act as immunomodulators, activating immune cells and receptors and functioning as broad-spectrum antimicrobial peptides (AMPs). These AMPs can target multidrug-resistant Gram-negative and Gram-positive bacteria, disrupt the cell membrane of pathogenic fungi, and even inhibit viral replication. Other peptides may employ diverse antimicrobial mechanisms such as generating oxidative stress via reactive oxygen molecules, producing lysozyme-like enzymes to degrade microbial cell walls, and promoting agglutination to prevent bacterial spread[17].

Our process may also facilitate the regeneration of new embryonic cells using the embryonic peri-vitelline fluid (PVF) to support tissue differentiation and regeneration applications[18].

Conclusion

A consistent, unlimited, supply of laboratory produced horseshoe crab amebocytes, and associated derivatives can help stabilize supply and extend the utility of LAL and related products by addressing both short and long-term concerns related to sustainability, innovation, and global harmonization. AG-cultured amebocytes represent a highly valuable and durable alternative to traditional LAL-based and recombinant endotoxin testing. Providing steady supply of consistent amebocytes will ease operational and regulatory pressures within the endotoxin detection industry, reduce costs for end-users, improve patient safety, and pave the way for new therapeutics and technologies.

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