USP Chapter <86> Bacterial Endotoxins Test Using Recombinant Reagents: A Step Forward, But Not a Milestone for Horseshoe Crab Conservation

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Abstract This paper evaluates the approval of the United States Pharmacopeia (USP) Chapter <86>, which introduces recombinant reagents as alternatives to *Limulus* Amebocyte Lysate (LAL) and *Tachypleus* Amebocyte Lysate (TAL) for bacterial endotoxins testing (BET). While this development marks progress in reducing dependence on horseshoe crab hemolymph for reagent production, it does not represent a significant breakthrough for horseshoe crab conservation. The primary threats to the world's four horseshoe crab species—habitat loss, unregulated economically driven harvesting in Asia, as well as other anthropogenic factors—remain largely unaddressed by the transition to recombinant reagents. Although these threats may be somewhat mitigated locally, the shift to recombinant alternatives does not sufficiently tackle the broader conservation challenges. This paper also examines the global pharmacopeial landscape, identifies barriers to industry adoption of recombinant reagents, and explains why the implementation of USP Chapter <86>, while a commendable step, falls short of making a meaningful impact on the overarching conservation issues confronting horseshoe crabs.

Keywords animal-free bacterial endotoxin testing, horseshoe crab conservation, *Limulus* Amebocyte Lysate (LAL), pharmaceutical supply chain sustainability, recombinant Cascade Reagent (rCR), recombinant Factor C (rFC), *Tachypleus* Amebocyte Lysate (TAL), USP Chapter <86>.

Introduction

The world's four extant horseshoe crab species—*Limulus polyphemus* (American horseshoe crab), found along the Atlantic coast of the United States from the Gulf of Maine to the northeastern Gulf of México and Yucatán Peninsula, and *Tachypleus tridentatus* (tri-spine horseshoe crab), *Tachypleus gigas* (coastal horseshoe crab), and *Carcinoscorpius rotundicauda* (mangrove horseshoe crab), found in coastal South and Southeast Asia—each exhibit genetic variation throughout their spawning ranges. These species are threatened by habitat loss—both marine and terrestrial—and various anthropogenic activities. The three Asian species face the threat of local extinction, driven by unregulated exploitation that varies across their range and widespread habitat destruction.

In contrast, *Limulus polyphemus*, classified by the International Union for Conservation of Nature (IUCN) Red List as Vulnerable in 2016, has benefitted from extensive management conservation efforts in the United States (ASMFC, 2024; Smith et al., 2016; Smith et al., 2023). However, certain regional populations, particularly in New York, continue to experience declines, necessitating further habitat protection as well as additional local harvest regulation (Botton et al., 2022a; Gauvry et al., 2022; Smith et al., 2016, Smith et al., 2023). Insufficient monitoring in the northeastern Gulf of México and Yucatán Peninsula, along with under-regulated marine life harvest in parts of Florida, raises additional concerns for *Limulus polyphemus* in those areas. While harvest pressures receive significant attention, the primary long-term threat to all four species remains habitat loss driven by coastal development and sea-level rise.

The Ecological Research & Development Group Inc. (ERDG) welcomes the approval of USP Chapter <86>, "Bacterial Endotoxins Test Using Recombinant Reagents," which provides animal-free alternatives to the widely used *Limulus* Amebocyte Lysate (LAL) and *Tachypleus* Amebocyte Lysate (TAL) for bacterial endotoxins testing (BET). This advancement represents significant progress in offering options that reduce reliance on horseshoe crabs for biomedical applications (USP, 2024a). However, ERDG contends that this is not a substantial milestone for global horseshoe crab conservation, especially for the three Asian species that face severe threats

such as habitat loss, unregulated harvesting, and commercial pressures—challenges not mitigated by the adoption of recombinant reagents (Gauvry, 2015; Gauvry et al., 2022).

USP Chapter <86>, set for official adoption in May 2025, introduces recombinant Factor C (rFC) and recombinant Cascade Reagent (rCR) as alternatives to traditional U.S. Food and Drug Administration (FDA) licensed reagents derived from the lysed amebocyte blood cells in the hemolymph of *Limulus polyphemus*, *Tachypleus tridentatus*, and *Tachypleus gigas*. These recombinant reagents are anticipated to play a crucial role in testing of human and veterinary medicines, ensuring conformance to safety standards while offering a non-lysate test reagent alternative (USP, 2024a). Although providing a non-lysate option to the pharmaceutical industry signifies a major step in decreasing the demand for horseshoe crabs, it alone does not address the broader threats to their survival, such as habitat degradation and unregulated harvesting. To ensure the long-term viability of these species, conservation efforts must continue to prioritize these more extensive issues.

Horseshoe Crab Harvesting in the U.S. and Asia: Stark Differences

U.S. Harvesting: A Managed Practice

In the U.S., horseshoe crab populations are managed with a focus on population growth and sustainability under the oversight of the Atlantic States Marine Fisheries Commission (ASMFC), a federal body responsible for regulating animals for both LAL production and bait harvesting. In 2022, approximately 911,826 *Limulus polyphemus* were collected for LAL production, with an estimated mortality rate of 15% (approx. 145,920 crabs). In the same year 570,988 crabs were harvested for use as bait in the whelk and eel fisheries, a practice that intentionally results in 100% mortality (ASMFC, 2024).

State-by-state harvesting quotas are set by the ASMFC, and some states have imposed additional restrictions, including harvesting bans, to protect local populations. These combined efforts have led to stable or growing populations of American horseshoe crabs throughout much of their spawning range along the east coast of the United States, with a focus on future sustainability. Even though bait harvesting is regulated to ensure sustainability and reduce excessive loss of life

compared to unregulated practices, the intentional sacrifice of such a large number of horseshoe crabs for bait remains deeply troubling.

However, there remains ongoing debate over the effectiveness of ASMFC's management practices, particularly regarding the unintentional mortality rate associated with LAL production. While some conservationists raise concerns about the impact of biomedical collection, it is important to note that the greatest threats to *Limulus polyphemus* stem from habitat loss—both marine and terrestrial—commercial fisheries discards, and other anthropogenic pressures (ASMFC, 2024). Thus, while the adoption of recombinant reagents may reduce the need for LAL collection and its associated mortality, it will not address the primary threats to the population that impede growth and influence management decisions. Conservation efforts must focus on these broader issues, rather than becoming complacent with recombinant reagents as if they resolve a major part of the conservation challenge (Gauvry, 2015; Gauvry, et al, 2022).

Asia: Unregulated Harvest and 100% Mortality

In contrast, the situation in Asia is far more dire, with horseshoe crab harvesting driven primarily by economic incentives rather than biomedical use. The three Asian species are heavily targeted because they are easy to collect and highly profitable. Harvesters rent two of the species *Tachypleus tridentatus* and *Tachypleus gigas* to TAL producers who bleed the horseshoe crabs for their amebocyte lysate before returning them to the harvesters. Subsequently, these harvested crabs along with the others are sold into secondary markets for human consumption, chitin production, fertilizers, and traditional medicine, resulting in 100% mortality for each harvested crab (Gauvry, 2015; Gauvry et al., 2022; Laurie et al., 2019). A very small number of TAL producers release horseshoe crabs back into the wild, the prevalent practice of renting crabs for TAL production effectively subsidizes harvesters' profit margins and makes TAL producers complicit in the decline of horseshoe crab populations.

Unlike the U.S., where harvesting is regulated under stringent sustainability practices, there are no meaningful regulations or effective enforcement mechanisms in Asia to manage horseshoe crab populations. The unregulated, economically driven market, fueled by poverty, livelihoods,

social status, and corporate greed, ensures that even with the introduction of recombinant reagents, these species will continue to face unsustainable exploitation (Botton et al., 2022a; Gauvry, 2015; Gauvry, et al., 2022).

The three Asian species face varying levels of risk, with *Tachypleus tridentatus* listed as endangered (Laurie et al., 2019). The other two species, *Tachypleus gigas* and *Carcinoscorpius rotundicauda*, are currently listed as data deficient by the IUCN (IUCN, 2024). While it is premature to assign a conservation status to these two species without formal data review, most studies indicate moderate to severe threats to their local populations, exacerbated by a lack of genetic connectivity among populations. In addition to overharvesting, the greatest threat to Asian horseshoe crabs is the loss of their spawning habitat—both marine and terrestrial—from coastal development, land reclamation, sea-level rise, and other anthropogenic factors, further accelerating population decline.

Unless governments in the region coordinate conservation efforts and address the economic, social, cultural, and corporate indifference to unsustainable harvesting and habitat loss, these species will continue to lose critical habitat, genetic diversity, and overall population viability (Botton et al., 2022a; Gauvry, 2015; Gauvry, et al., 2022, Laurie et al., 2019).

Therefore, USP Chapter <86> will likely have marginal impact on the Asian horseshoe crab population unless accompanied by efforts to reduce other factors that will make it less profitable to harvest them.

Impact of Testing Modalities: Kinetic vs. Gel Clot

Endotoxin testing methods vary widely across the globe. In the U.S. and Europe, harmonized compendial kinetic tests, such as the more sensitive and quantitative kinetic chromogenic and kinetic turbidimetric methods (USP 2024b), are preferred for bacterial endotoxin testing (BET). These methods require the use of instrumentation and computers that require validation and maintenance to achieve a test result. Those users who are familiar with the instrumentation and software are generally open to new test methods requiring either the same or different instrumentation. In contrast, the compendial LAL/TAL gel clot test (USP 2024b) remains

dominant in Asia, South America, and Africa. This method is simpler, requires no advanced instrumentation, and is far more affordable, making it the method of choice in less developed regions (Eckford, 2024). Although TAL producers also market kinetic chromogenic and turbidimetric lysate reagents to clients who request them, they may not have access to patented and approved recombinant alternatives to offer their customers who are currently using an instrumented assay. As a result, the reliance on harvesting and bleeding Asian horseshoe crabs for TAL will persist. The gel clot method, with its lower cost and simplicity, will likely continue to dominate in regions where more advanced testing infrastructure and support are not available.

Global Pharmacopoeial Landscape: European Pharmacopoeia vs. USP Chapter <86>

The European Pharmacopoeia (EP) has allowed the use of recombinant Factor C (rFC) since July 2020 under General Chapter 2.6.32 (EP, 2024). While Chapter 2.6.32 allows for the use of rFC, which contains only the first zymogen in the clotting cascade, it does not include information on recombinant cascade reagents (rCR), which use all three proteins from the clotting cascade, further narrowing its scope of recombinant options.

USP Chapter <86>, scheduled for adoption in May 2025, allows for the use of recombinant reagents but in addition to rFC, Chapter <86> provides guidance on the use of rCR reagents. USP Chapter <86> also requires that the user either provide validation of the test method or examine the manufacturer's primary validation package to assure that the method is appropriate for testing prior to using it routinely.

Both Chapter 2.6.32 and Chapter <86> follow the harmonized Endotoxins Test Chapter (USP Chapter <85>) with respect to standard curve preparation, and both require product-specific suitability testing (test for interfering factors) to verify that the validated method is appropriate for the product under test. Both chapters provide additional information on the use of rFC, which is a fluorometric method that is not described in Chapter <85>.

Lysate-Based vs. Recombinant Reagents

Lysate-based reagents contain all three zymogen proteins of the horseshoe crab clotting cascade: Factor C, Factor B, and the Proclotting Enzyme. Recombinant reagents, such as those currently marketed, use cloned enzymes from different horseshoe crab species (*Carcinoscorpius rotundicauda, Tachypleus tridentatus*, and *Limulus polyphemus)*, expressed using various mammalian and non-mammalian cells (Buchberger, et al, 2012; Ding & Bo,1994; Mizumura, et al, 2012). Recombinant Factor C (rFC) products use only the recombinant version of Factor C, the first enzyme in the cascade, while recombinant Cascade Reagent (rCR) products incorporate all three proteins. Importantly, none of these recombinant reagents includes the "Factor G" pathway present in lysate-based reagents, which prevents enhanced results or even false positives caused by possible glucan contamination in samples (Loverock, et al, 2010).

Global Adoption and Harmonization Challenges

Most of the world's major pharmacopeias are working toward writing and publishing chapters on the use of alternative endotoxin detection methods, including recombinant reagents. However, these chapters are not yet fully harmonized. For example, while the European Pharmacopoeia Chapter 2.6.32 allows for the use of recombinant Factor C, it does not provide for recombinant Cascade Reagents (rCR). The Indian and Korean pharmacopeias are expected to align with the USP, while the Chinese and Japanese pharmacopeias are still debating how to proceed (G. Gauvry, personal communication). The inclusion of a separate chapter for recombinant reagents reflects the distinct properties of these reagents compared to lysate-based methods.

As data become available through regulatory agencies and peer-reviewed publications, a greater understanding of recombinant methods will emerge. This will help ease any concerns that users may have and will support the broader adoption of recombinant reagents. However, until these reagents can demonstrate consistent equivalence across diverse applications, or until they are required by pharmacopeial product monographs, their widespread use will likely remain under utilized.

Challenges to Industry Adoption of Recombinant Reagents

The pharmaceutical industry's reluctance to fully adopt recombinant reagents can be attributed to several factors.

1. Patient Safety and Equivalency

Ensuring patient safety is the industry's top priority. Recombinant Factor C (rFC) and recombinant Cascade Reagent (rCR) offer certain advantages, such as non-reactivity to glucan contamination, which can cause false positives in traditional lysate-based methods. However, despite these benefits, recombinant reagents still require further validation to match the sensitivity, reliability, equivalence, and proven track record of LAL in detecting endotoxins (USP 2024c; USP 2024d). The transition to alternative reagents must be approached cautiously, as any deviation from established safety standards could pose risks to patient health.

2. Cost and Infrastructure

The cost of transitioning to recombinant reagents presents a significant barrier for many companies. Recombinant reagents are generally more expensive than their lysate-based counterparts, and shifting to these methods often requires the purchase of new laboratory equipment or requalification of existing testing systems. This financial challenge is particularly acute in regions such as Asia, where laboratories may rely heavily on the gel clot lysate test due to its affordability and the lack of advanced infrastructure needed for kinetic testing.

In addition to the direct costs, companies with large legacy portfolios face the added burden of preparing regulatory submissions for existing products. These submissions can be a significant expense, further complicating the industry's willingness to adopt recombinant methods (Eckford, 2024).

3. FDA Decision not to License Recombinant Reagents

While the USP has approved a new chapter on the use of recombinant reagents, the FDA has chosen not to regulate them as they currently do with lysate based reagents (CFR, 1973). This decision places the responsibility on individual companies to validate the safety and equivalence

of recombinant Factor C (rFC) and recombinant Cascade Reagent (rCR), as well as the vendor. This lack of FDA oversight adds complexity to the transition process, as companies must ensure that their internal validation processes meet the necessary Pharmaceutical Quality Management (PQMS) standards for patient safety and product integrity (Eckford, 2024).

It's important to recognize that the USP is a standards-setting organization, not a regulatory authority or auditor of a company's PQMS. While USP Chapter <86> provides a compendial option for companies to consider non-animal-based alternatives to LAL, it does not mandate their adoption. As a result, each user must conduct appropriate risk, impact, vendor and change management assessments to ensure the safety and equivalence of these alternatives for each product without the benefit of a required FDA license as a "safety net". Validation of alternative tests, execution of proper suitability screens, and ensuring equivalency with current LAL-based methods are critical steps for compliance with patient safety requirements (Eckford, 2024; USP, 2024a; USP 2024c; USP 2024d).

4. Historical Context and Industry Reluctance

The historical evolution from the Rabbit Pyrogen Test (RPT), first introduced to USP in 1942 (McCloskey, et al, 1943), to the LAL reagent, first recognized by the FDA in 1973 (CFR 1973), spanned 30 years. Complete adoption of the lysate test in lieu of the RPT did not happen until 14 years later with the publication of FDA's "Guideline on Validation of the *Limulus* Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices" (FDA, 1987, now retired). More recently, recombinant Factor C (rFC) was introduced in 2003, and despite its promise, it has been slow to gain traction due to high costs, relatively low sensitivity at the time of its introduction, its non-compendial status, and the FDA's reluctance to regulate its manufacture. This history demonstrates that while progress in bacterial endotoxin detection is often slow, it is ultimately achievable. However, the reluctance to fully transition to new methods, particularly in an industry that prioritizes safety and regulatory compliance, underscores the need for thorough validation and peer-reviewed studies that demonstrate equivalency with current lysate-based methods (USP, 2024a; Eckford, 2024)

The Role of the Pharmaceutical Industry in Catalyzing Change

The pharmaceutical industry has the potential to be an example and drive transformative change in endotoxins testing by providing data that will support subsequent decisions to move away from animal-derived reagents, including the use of recombinant reagents. However, this transition must be guided by two fundamental principles: the prioritization of patient safety and the development of reliable alternatives that meet the rigorous standards required for medical products worldwide.

While recombinant Factor C (rFC) and recombinant Cascade Reagent (rCR) offer promising alternatives to traditional *Limulus* Amebocyte Lysate (LAL) and *Tachypleus* Amebocyte Lysate (TAL) tests, their widespread adoption will depend on robust validation and evidence that they perform equivalently to lysate-based methods. Peer-reviewed studies demonstrating their reliability, efficacy, and safety are critical in convincing laboratory managers and regulators alike to embrace these alternatives.

Conclusion: A Step Forward, But Not a Milestone

While USP Chapter <86> represents progress toward reducing reliance on horseshoe crabs for bacterial endotoxin testing, it is not a milestone for horseshoe crab conservation. The adoption of recombinant reagents such as recombinant Factor C (rFC) and recombinant Cascade Reagent (rCR) faces significant challenges in both the U.S. and Asia. Regulatory hurdles, economic barriers, entrenched testing practices, and the prevalence of the gel clot method in less developed regions hinder the immediate widespread use of these alternatives.

More critically, the broader threats to horseshoe crab populations—habitat loss, unregulated and unsustainable harvesting, and economic pressures, especially in Asia—are not addressed by the adoption of recombinant reagents alone. Horseshoe crab populations in Asia remain vulnerable to exploitation, with unsustainable practices driven by economic incentives beyond the scope of endotoxin testing. Without concerted conservation efforts, these species will continue to face significant declines, even if the demand for LAL and TAL is reduced.

Thus, while USP Chapter <86> offers a path forward in bacterial endotoxin testing, it is important to manage expectations. It is a positive step toward reducing the use of animal-derived reagents, but it is not a definitive solution for horseshoe crab conservation. The pharmaceutical industry must continue to seek advancements in alternative bacterial endotoxin testing and adopt non-animal-based methods where feasible.

Moreover, effective supply chain management is crucial to addressing the unregulated harvesting practices in Asia. This includes divesting from TAL producers who do not adhere to best management practices that mirror or exceed those of LAL producers. Additionally, endorsing the Pharmaceutical Supply Chain Initiative (PSCI) which represents 74 of the world's largest pharmaceutical companies and their suppliers can significantly impact conservation efforts. The PSCI pledges to protect all endangered species and ceasing further TAL collection from *Tachypleus tridentatus* and *Tachypleus gigas*. By committing to no further collections from these species and discontinuing TAL use once existing supplies are exhausted, PSCI members can help alleviate commercial pressures on these vulnerable populations (PSCI, 2023).

Until these broader challenges are addressed, recombinant methods will not be the milestone that conservationists hope for. Comprehensive strategies that include regulatory reforms, ethical supply chain practices, and industry-wide commitments are essential to ensure the long-term viability of all four horseshoe crab species.

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