# Drivers of Implementation of Recombinant Technologies for Endotoxin Testing

As documented by the most recent US Pharmacopeia announcement,[1] the adoption of recombinant technologies for endotoxin testing is gaining momentum in the pharmaceutical industry. This article explores the fundamentals of using recombinant Cascade Reagents (rCR), their analytical performance and equivalency to LAL reagents, current developments within the regulatory framework (specifically updates provided by the USP and the factors accelerating their implementation.)

## Principles of Bacterial Endotoxin Testing

The bacterial endotoxin test (BET) is an enzymatic assay crucial for detecting minute quantities of bacterial endotoxins down to 0.005 EU/mL or even 0.001 EU/mL, if applicable. BET is used daily as a quality control test ensuring the safety of biopharmaceuticals and medical devices. Despite its importance, BET predominantly relies on reagents prepared from marine arthropods, in the US, the horseshoe crabs Limulus polyphemus.

## Traditional and Recombinant Endotoxin Testing Methods

These days, endotoxin testing employs two primary methods: the traditional Limulus Amebocyte Lysate (LAL) reagents and recombinant reagents which include two options: recombinant Cascade Reagents and recombinant Factor C reagents.

### *Traditional LAL Reagents*

Traditional LAL reagents utilize the blood clotting mechanism of horseshoe crabs. The hemolymph of these crabs contains granular blood cells (amebocytes) filled with coagulation factors, including Factor C, Factor B, pro-clotting enzyme, and coagulogen. In the presence of endotoxins, Factor C triggers a clotting reaction which leads to a formation of blood clot (gel clot reagent) or a formation of turbidity (turbidimetric reagent) or a development of yellow color (chromogenic reagent).

### *Production of LAL Reagents*

The production of LAL reagents involves several stages:

1. **Collection:** The hemolymph is collected from horseshoe crabs
2. **Centrifugation:** The hemolymph is mixed and centrifuged to collect amebocytes as a supernatant.
3. **Lysis:** The amebocytes are subjected to lysis.
4. **Formulation/Fill:** The raw lysate is formulated with buffers/excipients and a chromogenic substrate before being filled into vials and lyophilized.
5. **QC testing of the LAL reagent:** against USP Reference Standard Endotoxin and reference lysate.

The hemolymph serving as raw material for LAL can be subjected only to a limited incoming material inspection, thus is considered non-GMP. The production is conducted under current Good Manufacturing Practice (cGMP) conditions in ACC's FDA-licensed facility for LAL manufacture, ISO 13485:2016 certified.

## Drivers for Recombinant Technology Adoption

Several factors advocate for the accelerated implementation of recombinant technologies in endotoxin testing.

### *Technical Improvements and Advancements*

Typically, innovations aim to produce accurate, reproducible, and consistent results while enhancing laboratory productivity and efficiency. Recombinant Cascade Reagents (rCRs), such as PyroSmart NextGen®, have shown just that: improvement in accuracy, reproducibility and specificity to endotoxins compared to traditional LAL reagents. When paired with automated liquid handle systems, laboratory efficiency and high throughput are also considerably enhanced.

### *Supply Chain Risks*

Over 70 million BET tests are performed annually, a trend that continues to grow with the ever-increasing and ageing global population. 90% of all BET tests are still conducted using LAL reagents. Growing restrictions to harvest horseshoe crabs as a result of local and federal laws, together with the growing demand, create a supply chain risk. Implementing rCR reduces our dependence on natural resources, and thus decreases the supply chain risk. Pharmaceutical Supply Chain Initiative (PSCI), representing 74 major pharmaceutical companies, has publicly called for transitioning from TAL reagents (derived from endangered Asian horseshoe crab Tachypleus sp.) to LAL or recombinant technologies to address conservation and welfare concerns.

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## From the Pioneers in Endotoxin and Beta-D-Glucan Testing

Fifty years ago, ACC revolutionized the endotoxin detection industry by pioneering LAL testing methodology. We continued to innovate with the first and only FDA-cleared and CE marked rapid screening test for invasive fungal infections (IFI), Fungitell®.

Today, we are recognized as an international leader in endotoxin and glucan detection. Our legacy and commitment to quality stands, as we continue to provide **Protection Through Detection**™.

Our latest offering, **PyroSmart NextGen®**, is a recombinant cascade reagent (rCR) that replicates the LAL enzyme cascade without the use of animal content.

Our global headquarters are located in East Falmouth, Massachusetts, USA. In Europe, our office in Knowsley, UK, established 30 years ago, is supported by our distribution warehouse in Nijverdal, NL.

ACC is certified to I.S. EN ISO 13485:2016 and ISO 13485:2016. 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 GMP-compliant, ISO-registered and DEA-licensed Contract Test Services (CTS) Laboratory. 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 studies/protocols.

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### *Social and Corporate Responsibility*

A growing number of investors have expressed their concern about corporate impacts on ecosystems and global dependencies on nature by joining the investor-led initiative Nature Action 100, which has more than 200 participants representing over US$ 28 trillion in assets under management or advice. The biotechnology/ pharmaceutical sector is one of eight systemically important sectors that the initiative has identified for initial investor engagement. Some pharmaceutical companies, such as Eli Lilly, have already transitioned to using recombinant technology, setting a precedent for the industry.

## Conservation of Horseshoe Crabs

The Atlantic States Marine Fisheries Commission (ASMFC) monitors horseshoe crab populations and has been reporting positive developments in their numbers in the US. For example, over 16 million mature female horseshoe crabs and 50 million mature male horseshoe crabs have been recorded in the Delaware Bay region alone. The horseshoe crab, an integral keystone species of the Delaware Bay ecosystem, is much depended upon by many other species.

## Recombinant Cascade Reagents: PyroSmart NextGen®

PyroSmart NextGen® is a reagent containing recombinant Factor C, Factor B and Pro-clotting enzyme co-lyophilized with chromogenic substrate. As such, PyroSmart NextGen® is a reagent for kinetic chromogenic technique. The kinetic chromogenic technique is described in the harmonized USP Chapter <85> Bacterial Endotoxins Test which specifies that the technique should be conducted using an LAL/ TAL reagent.[2] Therefore, while the kinetic chromogenic technique is compendial, the reagent used for the technique is considered an alternative reagent to LAL, when using it to test a compendial article per USP monograph.

### *Development and Validation*

The development of PyroSmart NextGen® began in 2010, with its first iteration launched in Japan in 2015. The new generation reagent, launched globally in 2021, has been evaluated in numerous comparability studies.[3-7] To date, a total of nine peer-reviewed scientific publications, documenting the development, first generation PyroSmart®, two publications on method validation output of PyroSmart NextGen® and three comparability studies (https://www.acciusa.com/tools-and-resources/educational-content/acc-rcr-reference-list) were published.

### *Production of PyroSmart NextGen®*

The production of LAL reagents involves several stages:

1. **Expression/Purification of recombinant proteins in a bioreactor:** the genes coding the factors were cloned based on *Limulus polyphemus genome*.
2. **QC testing of the recombinant proteins:** subjected to the same requirements as for production of recombinant therapeutic proteins

3. **Formulation/Fill:** The recombinant proteins are formulated with buffers/excipients and a chromogenic substrate.
4. **Fill/Lyophilization:** 1 vial provides enough of reagent for ½ of 96well microplate.
5. **QC testing of the final reagent:** subjected to the same QC testing as LAL reagents but with more stringent specifications.

Start to finish, PyroSmart NextGen® production is conducted under cGMP conditions in ACC FDA-licensed facility for LAL manufacturing and ISO 13485:2016 certified. FDA Master File for PyroSmart NextGen® is in preparation.

### *Precision and Accuracy*

Precision is often expressed as repeatability, intermediate precision, and reproducibility. PyroSmart NextGen® has shown precision within acceptable values. For example, in Stevens et al, 2022, Correlation of Variation % based on endotoxin concentration was obtained within 20-35%.[3] A separate study by Kelley et al, 2023 showed that PyroSmart NextGen® had tighter precision than concurrently performed Pyrochrome when testing sodium citrate injection for endotoxin.[5]

### *Endotoxin Specificity*

PyroSmart NextGen® was shown in multiple studies to detect only endotoxins, including endotoxins varying by the structure of the Lipid A part of the lipopolysaccharide.[3] Unlike all LAL reagents, PyroSmart NextGen® does not produce a response to 1,3-β-glucans which either mimic the reactivity of endotoxin (in its absence, causing false positive results) or have a synergistic affect with the present endotoxin (causing enhanced results). In Stevens et al, 2022, two series of Reference Standard Endotoxin (RSE) concentrations were prepared; one spiked with 200 pg/mL glucan (which is considered to be a significant concentration highly likely to cause a false positive response with the LAL reagents). PyroSmart NextGen showed no difference between the RSE and RSE + glucan standard curves, confirming its specificity to endotoxins.[3]

### *High degree of Linearity and Accuracy*

High degree of linearity was confirmed in multiple publications and studies. Of note is Stevens et al. 2022 which included 24 onset times assays over three days, with each concentration analyzed in eight replicates, where the correlation coefficients ranged between 0.996 and 0.999, demonstrating a high degree of linearity and robustness.[3] The improved degree in linearity of standard curves generated by PyroSmart NextGen® leads to a high degree of accuracy: in absorbance microplate readers was determined as 71 to 140% for a wide range curve of 50 – 0.005 EU/mL (across different analysts and facilities, in 24 assays using eight replicates per concentration over three days).

### *Suitability for a range of sample matrixes*

The evidence of suitability of PyroSmart NextGen® with a wide range of drug products has already been documented and continues to grow with the implementation of this reagent in the field. For instance, Stevens et al, 2022 tested 27 different finished products (injectables), showing equivalent suitability and improved suitability with some products.[3]

## Comparison with LAL Reagents

### *Lot to lot reproducibility*

PyroSmart NextGen® demonstrates significantly less variability compared to traditional LAL reagents. In a comparison of randomly selected eight consecutive lots of LAL kinetic chromogenic reagents, the obtained onset times of individual standard concentrations varied lot to lot by 10% and the potencies ranged from 11 to 19 EU/ng. PyroSmart NextGen®, however, showed the variability between onset times of individual contractions lot to lot as low as 1% up to 4%, indicating greater reproducibility of its enzymatic rate.

### *Performance and Equivalence*

PyroSmart NextGen® has been demonstrated to be equivalent or superior in performance compared to traditional LAL reagents in multiple studies.[4-7] The reagent is a kinetic chromogenic assay, identical to traditional LAL chromogenic reagents but produced recombinantly. It offers high lot-to-lot reproducibility and precise results, with minimal sensitivity to 1,3-β-glucans, making it an easy to implement QC test.

## Phased Approach for Implementation of PyroSmart NextGen®

The USP Microbiology Expert Committee has approved the inclusion of Chapter *<86> Bacterial Endotoxins Test using Recombinant Reagents* to the US Pharmacopeia – National Formulary (USP-NF).[8] The final text will be published for early adoption on 01 November 2025 and will become official on 01 MAY 2025.

ACC follows a phased approach for implementing PyroSmart NextGen:

- **Phase 1:** Convert testing of low-risk sample types from LAL to PyroSmart NextGen® to immediately to mitigate the supply chain risk: pharmaceutical grade water,[9] water for injection, pharmaceutical ingredients, buffers and solutions, cell culture media. Water testing for endotoxin constitutes between 70 – 80% of global samples daily. This presents a low regulatory threshold and may be subject to annual reporting only.
- **Phase 2:** Early implementation of USP <86> for expanding the testing to final products tested per USP monographs

following the requirements of USP <86> to verify suitability for the intended purpose under actual conditions of use (per USP <1226>) and to document comparability to LAL, where required.[8,10]

## Conclusion

Adopting recombinant technologies for endotoxin testing is driven by technical advancements, supply chain risk mitigation, social and corporate responsibility, and conservation efforts. Recombinant Cascade Reagents, such as PyroSmart NextGen® offer a sustainable and reliable alternative to traditional LAL reagents: same compendial kinetic chromogenic technique, but with documented improvements. PyroSmart NextGen® is fully manufactured under cGMP conditions in FDA-licensed facility for LAL manufacturing, ISO 13485 certified, thus meets and exceeds quality requirements for the production of LAL. This high level of quality provides ensurances for accurate and reproducible results while reducing the reliance on natural resources. The pharmaceutical industry must accelerate implementation of innovations in BET to reduce the global dependency on natural resources (such as the horseshoe crabs) and to advance the specificity and reproducibility of BET assays.

## References


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9. *USP <1231> Water for Pharmaceutical Purposes*


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