American Pharmaceutical Review™

Endotoxin Detection Part XI: Ahead of the Curve

Testing Colored Samples, Validation Process - Recombinant Cascade Reagent (rCR), The Horseshoe Crab Population and Automation Solutions for BET

A Supplement to American Pharmaceutical Review

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ASSOCIATES OF CAPE COD INCORPORATED

Specialists in Endotoxin and Glucan Detection

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Specializing in chromogenic and turbidimetric reagent technologies, Associates of Cape Cod, Inc. (ACC) has been a global leader in endotoxin and (1→3)-ß-D-glucans detection products and services for nearly 50 years. ACC pioneered LAL testing methodology and was the first FDA licensed company to manufacture LAL reagents, and throughout the years has grown to be an internationally recognized leader in endotoxin detection.

In 2021, we were very excited to introduce another first when we launched the first commercially available sustainable BET Recombinant Cascade Reagent (rCR), PyroSmart NextGen®. PyroSmart NextGen® is completely horseshoe crab blood free and unlike first generation recombinant BET reagents (rFC), PyroSmart NextGen® uses the same LAL cascade as traditional LAL reagents, while eliminating the potential for (1→3)-ß-D-glucans. This eliminates the need to change test methods and purchase new specialized instrumentation as required by first generation rFC recombinant reagents. Simply put… same Instrument, same preparation steps, same method. Furthermore, with the inclusion of rCR recombinant reagents being added to USP Chapter 86, there's never been a better time to consider transitioning to PyroSmart NextGen®. **Keep your Method… Make an Impact!**

Our worldwide headquarters are located in Falmouth, Massachusetts. With a dedication to quality, ACC is certified to I.S. EN ISO 13485:2016 and ISO 13485:2016. We are FDA Inspected and operate DEA Licensed and CLIA-certified laboratories. Our endotoxin detection reagents, instruments and software are used within the Pharmaceutical, Medical-Device, Biotechnology, Compounding Pharmacy and Dialysis industries for quality control, product release and research. 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 Contract Test Services (CTS) Laboratory which has specialized in testing for endotoxin and glucan contamination for over 40 years. Our CTS laboratory is GMP compliant, ISO registered and DEA licensed and is capable of handling all controlled drug substances except those included in Schedule 1. All testing services can be performed to FDA, USP, EP and/or JP regulatory guidelines. 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 (LER) studies/protocols.

ACC also offers a clinical diagnostic product line – Fungitell® and operates a CLIA-certified laboratory specializing in (1→3)-ß-D-glucans analysis to support the diagnosis of Invasive Fungal Disease (IFD).

Endotoxin Detection Part XI

Ahead of the Curve: Testing Colored Samples, Validation Process - Recombinant Cascade Reagent (rCR), The Horseshoe Crab Population and Automation Solutions for BET

A Note from the Editor

Welcome to our eleventh supplement on Endotoxin Detection.

A lot has changed in the last year.

A year ago, the pandemic was still the biggest story. While COVID-19 still makes headlines, it is certainly not the dominating story it was then.

In fact, a recent survey conducted by YouGov using a nationally representative sample of 1,665 U.S. adults interviewed online from Aug. 17 to Aug. 21, 2023, found that just 7% of Americans now say they are very worried about getting COVID-19, down from 11% in early September 2022 and 13% in April 2022.

A larger number — 31% — say they are at least somewhat worried, although that number is down from 43% in September 2022 and 45% in April 2022.

The survey chalks up these findings to the fact that many Americans have been vaccinated, or have achieved some measure of immunity through infection, vaccination or a combination of both.

At this point we are well aware of the monumental effort the pharmaceutical industry took on to bring the first round of COVID-19 vaccines to market – and the efforts they continue to put forth to bring new vaccines to market as COVID-19 changes.

Even though Americans attitudes have changed regarding COVID-19, what hasn't changed is the pharmaceutical industry's need to continue to bring life-saving treatments to people the world over.

And, not just treatments, but safe treatments.

And that's where this supplement comes in.

As the pharmaceutical industry develops more advanced therapies the need to ensure their safety to patients does not diminish. The need to ensure products are free from any sort of contamination is critical before products leave the manufacturing facility.

The use of biopharmaceuticals continues to expand and as their popularity grows, so does the industry's need to test for bacterial endotoxins.

The goal of this supplement is to provide as much information as possible regarding current thinking and methodologies for endotoxin testing and removal.

As you look through these articles, we hope you gain valuable insight and knowledge regarding this industry critical topic.

If you have any questions or comments, please contact us.

Thanks again for reading,

Mike Auerbach

Editor in Chief

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The Myth of Testing Colored Samples: Debunked

Global Technical Services Team
Associates of Cape Cod, Inc.

We often hear from end users who are in the process of designing a suitability study for their new sample, "My sample is yellow, so my only choice of reagent is the turbidimetric test". But is that really the case?

Know Your Enemy: An In-depth Look at Bacterial Endotoxins

Bacterial endotoxins can be nasty little pests! As non-infectious particles found within the cell walls of every Gram-negative bacteria, endotoxins can induce immune responses leading to fever, inflammation, septic shock, and even death in severe cases. Contamination of pharmaceutical and healthcare products with endotoxins, therefore, poses serious risks. Rigorous in-house programs for endotoxin testing are imperative to ensure the safety and quality of pharmaceutical products and medical devices. As such, BET is a regulatory requirement and a critical step in safeguarding public health.

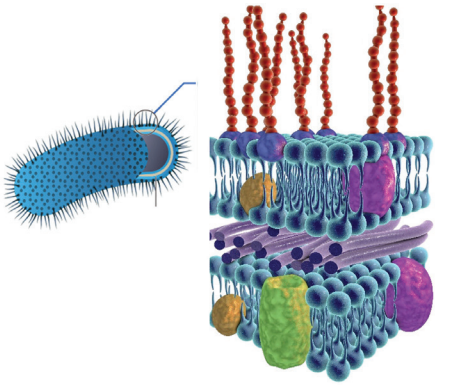


Figure 1. Diagram of cell envelope of a Gram-negative bacterial cell depicting the endotoxin structures in the outer membrane of the bacteria.

Color Me Curious: The Science Behind Chromogenic Testing, BET

Have you ever wondered how kinetic chromogenic testing works? Next, we'll walk you through the science behind this pharmacopeial technique, exploring how chromogenic tests

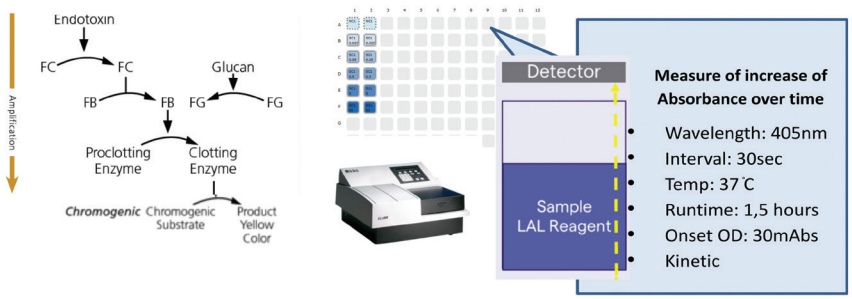


Figure 2. Depiction of the LAL cascade mechanism and the method principle of a kinetic chromogenic test.

measure the color change in a reaction to determine the presence of bacterial endotoxins.

The magic of chromogenic testing lies in its simplicity, linearity and accuracy. In addition to the LAL enzymes of the cascade mechanism (Factor C, Factor B and Preclotting enzyme), this test uses a chromogenic substrate.

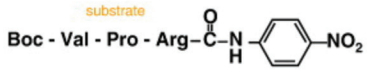


Figure 3. Example of Chromogenic substrate used in ACC chromogenic reagents.

The chromogenic substrate, which is colorless initially, is known to react with an activated Clotting Enzyme – as a result of Factor C activation by endotoxin. As Clotting enzyme cleaves the Arginine – CO bond in the chromogenic substrate, it releases a chromophore called para-nitroaniline (pNA) - a particle that absorbs light (with the absorption maxima close to the visible wavelength of 405nm) - and causes a change in color to yellow (as visible to the naked eye).

The resulting color change is then measured using an absorbance spectrophotometer. It was documented in the past that the intensity of the developing color is proportional to the amount of endotoxin present in the sample, allowing for a quantitative analysis of endotoxin in the sample.

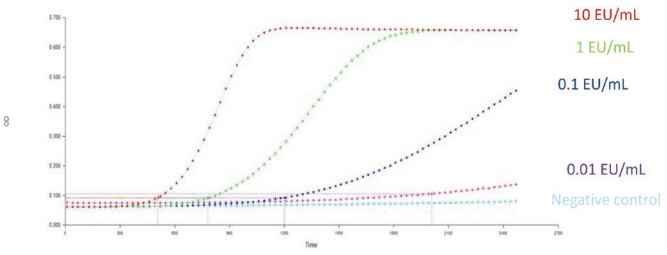


Figure 4. A typical diagram of the developing absorbance as measured at 405nm as dependent on endotoxin concentration.

It's a blend of biology and colorimetry that delivers rapid, accurate results, that made chromogenic testing a game-changer in the field of bacterial endotoxin testing back in 1990s.

How the Recombinant Chromogenic Test Further Improves the Output

The recombinant chromogenic test takes the advantages of the chromogenic test to the next level. How? Thanks to several groundbreaking features:

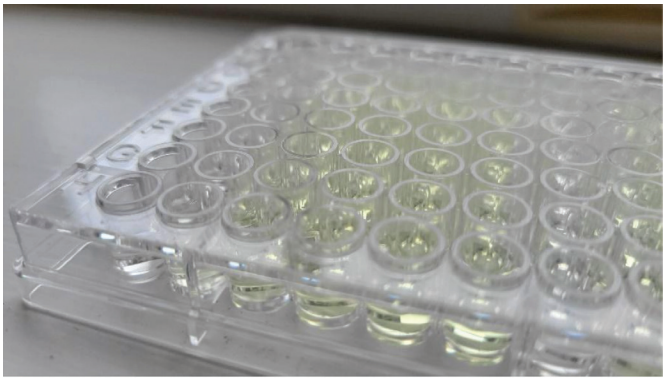


Figure 5. A typical microplate showing the developed color at the end of a kinetic chromogenic test.

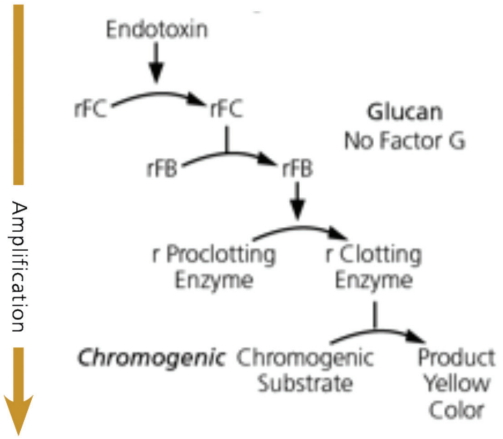


Figure 6. Mechanism of action of the recombinant cascade reagent.

- First and foremost, recombinant cascade reagent (rCR) PyroSmart NextGen® employs recombinant Factor C, Factor B and Proclotting enzyme, genetically cloned from Limulus polyphemus and expressed preparations of the cascade enzymes, thereby eliminating the need for animal-derived components and making the test eco-friendly.
- Furthermore, it is free of Factor G, a native component of the animal-derived LAL reagent, that is documented to be cause co-sensitivity to 1,3-β-glucans (common contaminants) thus reducing the risk of Out of Specification results.
- Perhaps most importantly, it has a documented lot-to-lot reproducibility of results which is a building stone towards standardization and modernization within the quality control laboratories (including automation of liquid handling).

At ACC, PyroSmart NextGen® is manufactured with consistent quality and performance under the same cGMP conditions in the same

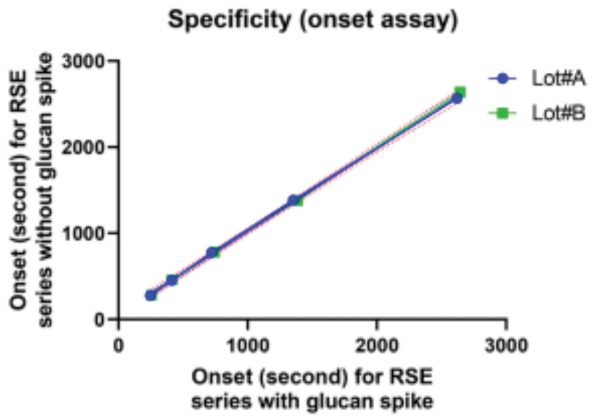


Figure 7. Linear regression of Onset Times (in seconds) for two RSE standard series with and without 1,3-β-glucan spike.

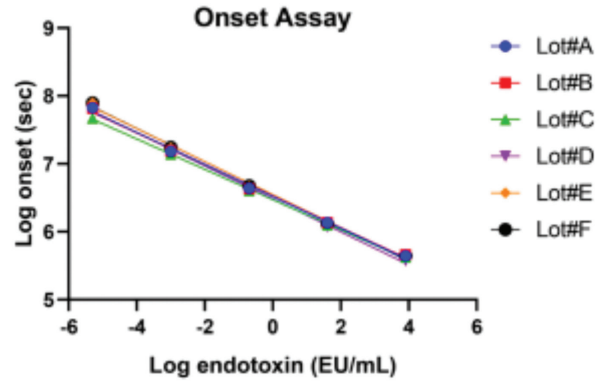


Figure 8. RSE standard curve series using six different lots of PyroSmart NextGen® demonstrating strong lot to lot reproducibility.

ISO 14385-certified facility as our FDA-licensed LAL reagent. This guarantees reliable and repeatable results, making rCR a robust and sustainable solution for endotoxin testing.

Debunking The Myth: The Data Behind Testing Colored Samples

There's a common misconception that chromogenic testing struggles when it comes to colored samples. Here, we'll set the record straight, showcasing the data that proves chromogenic testing, including the recombinant chromogenic assay, works efficiently and accurately on colored samples following a well-executed method suitability.

Per USP <85> and <1085>, method suitability testing is to be done on all samples prior to routine testing (1, 2). This allows for appropriate method development and it typically includes testing the sample in a series of dilutions (not exceeding the Maximum Valid Dilution) and evaluating the assay setup (reagent type, method type and instrumentation) for compatibility with the sample.

Fun fact #1: Most pharmaceutical sample types interfere with the BET.

Fun fact #2: A vast majority of sample interferences are overcome by simple dilution in water for BET (such as LAL Reagent Water (LRW)).

Colored samples are no different. Often in addition to the inherent color, they are likely to consist of components that interfere with the test. Based on our experience, dilution in LRW is highly likely to resolve both concerns – the optical and chemical interference - in one simple step.

In addition to dilution, there is another invaluable tool: instrumentation and software. The advent of advanced spectrophotometric methods has significantly alleviated the concern with testing colored samples. Baseline setting and zeroing play a pivotal role in this process. For example, in Pyros Kinetix Flex tube reader, where each well is individually timed and evaluated, it involves recording the initial absorbance of the sample. This is essentially measuring the absorbance by the inherent color of the sample before any reaction takes place. This baseline reading is then used as a reference point for all subsequent 10 second measurements, allowing the true color intensity increase to be accurately captured, irrespective of the color of the sample itself.

Pyros Kinetix Flex is powered by Pyros eXpress software which has built-in specifications for the raw data retrieved by each well. A sample with an intense color absorbing at 405nm will yield low transmittance values during the initial zeroing period and thus will be flagged in the software as being out of range, alarming the operator to take further action.

Case Studies: Real-World Applications of Chromogenic Methods for Colored Samples Testing

So how does this all work together? Let's examine comparability testing of MIC injection - a vitamin mix injection consisting of the primary compounds (methionine, inositol, choline) in addition to other components, e.g. Vitamin B12. Depending on the concentration of the components, the final preparation may look like this:

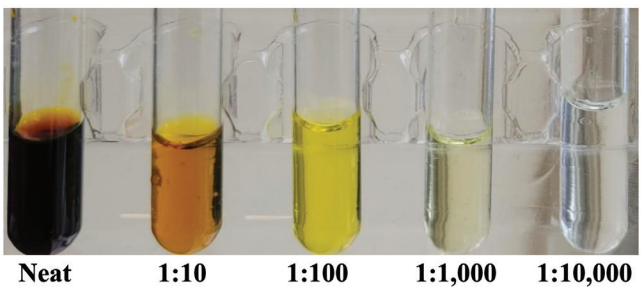


Figure 9. Dilution series of MIC injection

- Dilution series in LRW (MVD = 14,000)
- Addition of the BET reagent yields an additional dilution of the colored background.
- Testing the MIC injection using the kinetic turbidimetric assay (KTA):
  - Data collection plots for Positive control and Positive Product Controls for all dilutions tested (nominal value of 0.5 EU/mL):

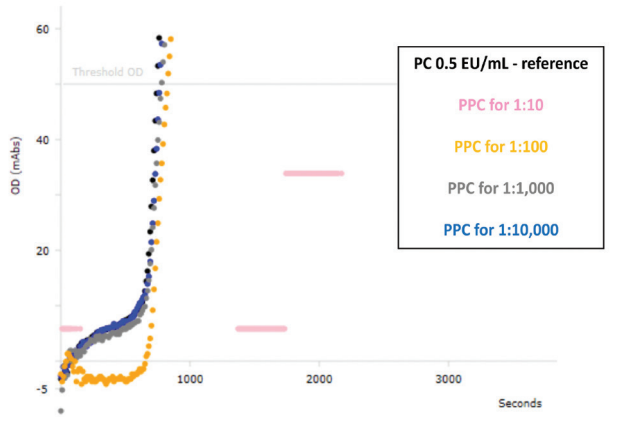


Figure 10. Data collection plots obtained for the range of MIC injection dilutions spiked with 0.5 EU/mL when tested by kinetic turbidimetric test in Pyros Kinetix® Flex tube reader.

  - Interpretation:
    - Neat – not tested. The concentrated MIC injection is off deep yellow color which absorbs non-specifically a full visible light spectrum.

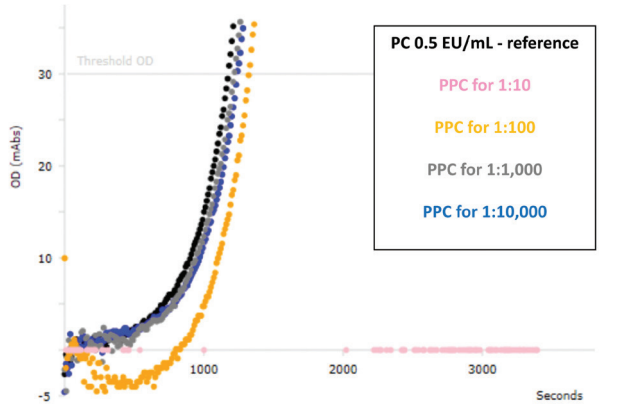


Figure 11. Data collection plots obtained for the range of MIC injection dilutions spiked with 0.5 EU/mL when tested by kinetic chromogenic test in Pyros Kinetix® Flex tube reader.

    - PPC for 1:10 – the inherent color is still too deep for the turbidimetric test, still absorbing the passing light non-specifically, Pyros eXpress notifies the user 60 seconds into the test that the transmittance specification was not met.
    - PPC for 1:100 – there is a residual optical interference between 0 to 800 seconds, which is then overcome by the increasing change in turbidity, related to the endotoxin reaction.
    - PPC for 1:1,000 – no optical interference.
    - PPC for 1:10,000 – no optical interference.

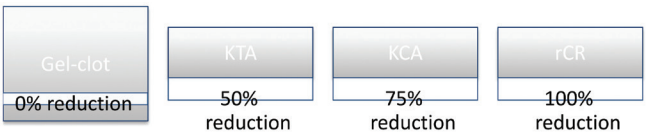
Table 1. Summary evaluation of the photometric data

	Dilutions	Mean EU/mL	PPC Recovery %	PPC CV%
KTA	1:10	Invalid	< 1%	Invalid
	1:100	< 0.5	82%	1.58
	1:1,000	<5	109%	0.39
	1:10,000	<50	109%	0.21
KCA	1:10	Invalid	< 1%	Invalid
	1:100	< 0.5	55%	0.61
	1:1,000	<5	85%	1.83
	1:10,000	<50	78%	1.42
rCR	1:500	< 2.5	90%	0.45

- In conclusion: the magnitude of optical interference observed on the turbidimetric test vs. chromogenic test was identical. Residual interference was observed at 1:100 dilution when testing by both methods. 1:1,000 dilution was free of both optical and chemical interference when testing by both LAL methods and thus could be chosen for further testing and validation.
- Additional testing was done with PyroSmart NextGen® where MIC injection was diluted 1:500 and that was sufficient enough to overcome the optical interference.

Expert Opinions: Quality Control Technicians Weigh In

Don't just take our word for it! Ask around! Leading pharma QC scientists and managers successfully validated kinetic chromogenic testing for colored samples. In some cases, they choose to go directly to the chromogenic technique, taking advantage of the wide dynamic range of the test, some started with the turbidimetric technique and then transitioned to the chromogenic one. Others, especially when implementing in-house testing for new products, go directly to the use of the recombinant chromogenic tests for colored samples. Apart from analytical performance, the photometric techniques comply with the



3R principles (Reduce, Replace, Recycle) in reducing the amount of the raw animal-derived material in the reagent with the recombinant reagent completely eliminating it:

All About Dilution: A Key to Unlocking Accurate Results

In closing, proper dilution techniques are instrumental in facilitating accurate results with colored samples (as with colorless ones), thus dismantling the misconception around chromogenic testing's capabilities. Understanding the components of the reaction and using the right instrumentation/software platforms with built-in features to report samples not meeting specifications allow the user to identify any issues shortly into the assay. With the appropriate method development, the chromogenic technique can be used for testing of colored samples with equivalent results to the turbidimetric technique.

The recombinant chromogenic method confirmed the validity of the results even at a lower dilution and has been proven suitable for colored samples as well. In the light of expert opinions, empirical data and the ethical commitment to animal welfare, it is evident that the recombinant chromogenic test is a robust, sustainable and reliable approach for endotoxin testing, regardless of sample color (3-7).

Embracing state-of-the-art methods signifies a leap forward in pharmaceutical quality control towards standardization and modernization of the procedures, while ensuring the safety and efficacy of medical products.

References

1. Bacterial Endotoxins Test <85>, United States Pharmacopeia (current revision), United States Pharmacopeial Convention, Rockville, MD.
2. Guidelines for Bacterial Endotoxins Test <1085>, United States Pharmacopeia (current revision), United States Pharmacopeial Convention, Rockville, MD.
3. Shapovalova O V et. al. New direction in the determination of bacterial endotoxins: Analysis using recombinant Factor C. Pharmaceutical chemistry journal, 56, 1133-1139, 2022.
4. Stevens I et. al. Advanced Recombinant Cascade Reagent PyroSmart NextGen® for Bacterial Endotoxins Test as Described in the Pharmacopeias BPB, Vol.5, No. 5 105-114 (2022)
5. Kelley M et. al. Evaluation of Recombinant Cascade Reagent PyroSmart NextGen® and Limulus Amebocyte Lysate Equivalency in a Plate and Tube Reader for Bacterial Endotoxins Testing, BPB, Vol.6, No. 1 11-15 (2023).
6. Kelley M et al. A Demonstration of the Validation Process for Alternative Endotoxin Testing Methods Using PyroSmart NextGen® Recombinant Cascade Reagent, BPB, Vol.6, No. 2 68-75 (2023).
7. Kikuchi Y et al. Collaborative study of bacterial endotoxins test using recombinant Factor C-based procedure for detection of lipopolysaccharides (Part 3). Pharmaceutical and Medical Device Regulatory Science, 54 (4), 341-351 (2023).

The Impact of Biomedical Use of Horseshoe Crabs

Post Pandemic Update

Brett Hoffmeister
LAL Manufacturing Manager
Associates of Cape Cod, Inc.

Introduction

The worldwide impact of the pandemic and the remarkable response to it, has changed the way we live, work and play. The Bacterial Endotoxins Test (BET) industry is not immune to the impact and looking back at the past several years it's easy to recall some of the concerns about the industry supply lines and ability of the BET industry to rise to the demands of the COVID-19 treatments and vaccine development. Also of concern was the horseshoe crab population in the U.S. as LAL manufacturers responded to the call by the pharmaceutical industry to supply this vital assay. We are proud to report that not a single vaccine nor treatment for COVID-19 was delayed for lack of BET reagents. In fact, the BET industry responded to an unprecedented time in the world's history and satisfied the demand from billions of vaccines being administered and countless treatment options being utilized. Supply lines were robust and met the many challenges as they are designed to do. Scalability of the industry was a key element in meeting the many demands placed on vaccine and treatment development, production and delivery.

Several published articles and reports contained information that is inaccurate regarding the horseshoe crab population and its use in the biomedical industry. Associates of Cape Cod Inc. (ACC) addressed some of some of the misleading suggestions made in those publications in 2018: Impact of Biomedical PR18-033 (acciusa.com) and again in working with the pharmaceutical industry in 2021: COVID-19 and the Sustainability of the LAL Supply (pda.org). With the impact of COVID-19 largely behind us it is an appropriate time to review the status of the American horseshoe crab, post pandemic, and address some of the most recent rumors regarding the true impact of the industry.

Recently and continually, it has been suggested that manufacturers of *Limulus* Amebocyte lysate (LAL) reagents are loosely regulated and a primary contributor to a speculative population decline of the American horseshoe crab, *Limulus polyphemus*.[1,6,10] This article will demonstrate that the overall number of horseshoe crabs is stable; *L. polyphemus* is certainly not in danger of extinction, and is thriving in many areas of its range. The LAL industry is closely regulated and has a relatively minor impact on mortality in the horseshoe crab population, even during an event such as a pandemic. In fact, it's a fair statement to say that in many areas where LAL manufacturers operate, the population of HSC in the US has actually grown in recent years.

Is the horseshoe crab population in the United States in decline? The simple answer is that the total number of horseshoe crabs is healthy, stable, and there is strong evidence it is increasing. There is reliable data concerning horseshoe crab population numbers and trends for over 20 years, and analysis of that data shows no evidence of an overall decline over that time. The American horseshoe crab has a widespread distribution made up of multiple populations along the east coast of the United States and horseshoe crabs populations appear to be stable and/or growing in most regions of the US.

The largest population of horseshoe crabs on the east coast is in Delaware Bay, and the most recent data show that numbers there are stable and/or increasing. A summary of an October 5, 2016, meeting of the Atlantic States Marine Fisheries Commission (ASMFC) Horseshoe Crab Technical Committee included estimates for horseshoe crabs in the Delaware Bay from 2015.[7] The collected data indicated the presence of approximately 8.1 million adult females and 16.4 million adult males, which is an increase from 7.9 million females and 15.2 million males in 2014.[4] Compare that to 2021 with an estimated population of 13.5 million adult females and 39 million adult males.[5] These data would indicate the population growing significantly, in less than 10 years in that region alone.

In the Northeast region, populations are smaller than further south. Variations in subpopulation sizes do not have a huge impact on the metapopulation; however, variations may be important locally. In New York, where there is a bait fishery but no biomedical fishery, surveying shows declining numbers. In Connecticut, where there is also a modest bait fishery but no biomedical fishery, populations have also declined. In this region management actions have included lunar closures, and prohibitions on harvesting spawning animals off the beach in an effort to protect spawning populations in hopes of reversing these trends.[2]

In Massachusetts, the most recent 2021 Compliance Report by the Massachusetts Division of Marine Fisheries (MADMF) for the ASMFC states: "Horseshoe crab survey results from the 2021 DMF spring and fall trawl surveys were mixed. South of Cape Cod, mean number and weight

of spring caught males and females in SNE remain near their respective time series highs, but at or below time series medians in the fall."[11]

The American horseshoe crab has distinct, fragmented subpopulations all over the eastern seaboard, including states north of Massachusetts. These northern fisheries are so small that these states are not required to report population trends to the ASMFC. Georgia and Florida both have horseshoe crab populations that appear to be stable.

Is biomedical fishery a significant contributor to horseshoe crab mortality?

The process of extracting the blood from the horseshoe crabs is minimally invasive and the overwhelming majority of crabs that undergo the procedure survive the process. In fact, many studies demonstrate survivability of the animals when treated properly and carefully. One study of nearly 70k crabs bled by biomedical companies over the course of years concluded the bled crabs survival rate was as good or better than the un-bled crabs.[12] The biomedical community supports and practices a release program where crabs caught under a biomedical license are released back to the wild. The Atlantic States Marine Fishery Commission (ASMFC), which manages the horseshoe crabs along the entire east coast, attributes 15% mortality to those released crabs,[2] assuming an 85% survival rate for use in the manufacture of LAL.

Horseshoe crabs are also used as bait in whelk and eel fisheries in the US and abroad. Horseshoe crabs are harvested and placed in traps where the eels or conch enter but cannot easily exit. This process is fatal to the crabs and has come under significant scrutiny coast wide with several states choosing to prohibit the practice altogether. In states that do allow a bait harvest, measures such as minimum sizes, a male only harvest and/or prohibitions around harvest timing help to protect spawning stock. Coast wide from Maine to Florida, a five year average of 735,000 crabs are caught annually to be used for bait. This is roughly one half of the coastwide quota of 1.5 million allowed to be harvested for bait. This number dropped significantly during the pandemic when restaurants closed or saw dramatic reductions in patrons seeking whelk derived products.[5]

The coast wide horseshoe crab mortality is represented in the graphs below. It can be seen that the biomedical industry does not substantially contribute to overall mortality (Figure 1).[5] The five year average estimated mortality for LAL manufacturing is ~ 94,000 crabs.

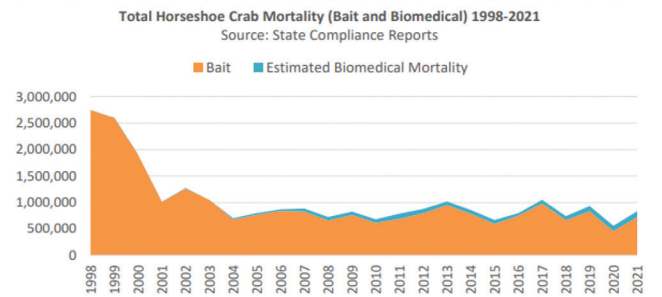


Figure 1.

To further evaluate if areas of biomedical catch and release are impacting horseshoe crab populations, an analysis of areas with biomedical manufacturers is explained below.

Horseshoe crabs are collected for biomedical purposes and for bait in the Delaware Bay and for biomedical purposes only in the coastal waters of South Carolina. Population numbers in all of these areas are either stable or increasing.

In Rhode Island, where there is a limited bait fishery and limited biomedical fishery, recent numbers are low, but data gave no indication of an increasing or decreasing trend.

In Massachusetts, where there is a biomedical fishery and a bait fishery, survey data show positive population trends, particularly in the southern region where the fisheries are concentrated. Massachusetts uses a combination of three surveys to gauge trends in the fishery; A trawl survey that is run in the Spring and Fall in waters south and North of Cape Cod, about a dozen beach surveys and market surveys capturing prosomal width of crabs sampled at both bait vendors and the biomedical facilities.

Most telling is the trawl survey which began in 1983 Shown below are data from the Massachusetts spring and fall surveys depicting male and female catches per tow. South of Cape cod where 85% of the fishery exists (Figure 2) and North of Cape Cod (Figure 3).[11]

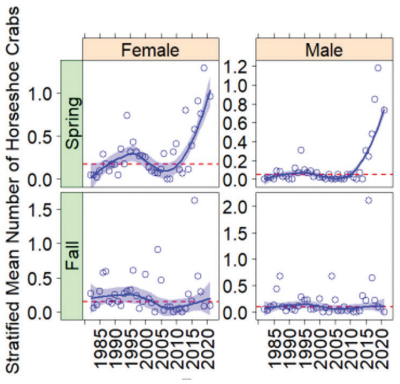


Figure 2.

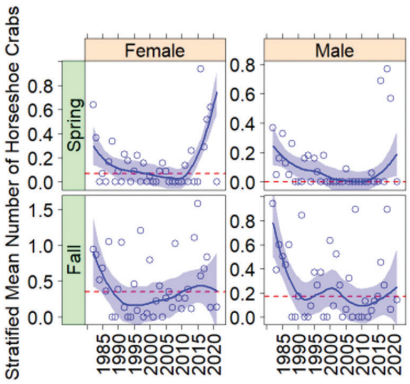


Figure 3.

In summary, these reports give no indication of a correlation between the biomedical fishery and population decline. In the regions with the largest biomedical fisheries, numbers are stable or even increasing.

Is the horseshoe crab fishery monitored?

All states which have a significant harvest of crabs for use in the bait and/or biomedical industry are required by law to report information regarding the number and sex of crabs harvested to the ASMFC.

For LAL manufacturing, data containing the sex, number, origin, and dead loss must be reported. That is to say that every crab that enters a LAL facility is counted, sexed and reported to the state. In turn the state reports these data to the ASMFC which then produces annual reports, guidelines and quotas for the coast wide fishery and individual states. States can use the quotas as a regulation or reduce but never increase the quota. Some have reduced or eliminated harvest for bait altogether.

Many states protect the populations of crabs by limiting access to spawning populations and or/ females. This is done with lunar closures prohibiting harvest at peak spawning time, delaying harvest till after peak spawning and requiring Male only fisheries for bait. Size limits can ensure mature animals are harvested.

It should be noted that LAL manufacturers are subject to audits and inspection by any number of entities including the US FDA, ISO, fisheries managers, law enforcement, customers, and potential customers among those that routinely visit on site. These can happen anytime with or without notice.

Additionally, the management of the fishery by the ASMFC and state fishery departments has ensured that the use of the horseshoe crabs in both bait and biomedical industries is closely monitored.

Media

Recent articles, podcasts and similar media often suggest that the handling of the horseshoe crabs results in a higher mortality than is estimated by the ASMFC.[6,10] In part because some experimental studies focused on the after effects of the simulated process on horseshoe crabs, that are handled and kept under more stressful conditions than those used by the LAL manufacturers. This resulted in reports of high mortality and sub-lethal effects. Like all animals, if horseshoe crabs are not treated well, they do not fare well. Proper handling of horseshoe crabs is important to maximize the survival of horseshoe crabs returned to the water. This is recognized by the ASMFC, who manages the horseshoe crab fishery according to a fishery management plan (FMP) A code of best management practices (BMPs) has been formulated between the ASMFC, the states, and biomedical companies, which builds on the catch and release practices that have been in place for many years. These BMP's were reviewed and updated by stakeholders and scientists in 2023.[3] In Massachusetts, there is a regulatory requirement to adhere to specific best practices as a condition of the biomedical license.

Summary

In conclusion: The management of horseshoe crabs is highly regulated in the United States on both a regional and state specific level. The population successfully supports both a bait industry as well as a LAL manufacturing industry and there is encouraging evidence that the population is growing in many areas. Though life has changed for many of us after the major impact of COVID-19 has begun to wane. At least one aspect, the overall population of horseshoe crabs in the US, is stable and/or increasing. This is of course good news and serves as evidence that responsible use and good management of the fishery pays dividends.

References

1. Anderson RL, Watson WH 3rd, Chabot CC. Sublethal behavioral and physiological effects of the biomedical bleeding process on the American horseshoe crab, Limulus polyphemus. Biol Bull. 2013; 225(3):137-51. https://www.ncbi.nlm.nih.gov/pubmed/24445440
2. ASMFC ATLANTIC STATES MARINE FISHERIES COMMISSION REVIEW OF THE INTERSTATE FISHERY MANAGEMENT PLAN November 2022. https://asmfc.org/uploads/file/63fe30fcHSC_FMP_Review_FY2021.pdf
3. ASMFC. Biomedical Best Management Practices work group May, 2023 https://asmfc.org/uploads/file/645bf065HSC_Biomedical_BMPs_2023.pdf
4. ASMFC Horseshoe Crab and Delaware Bay Ecosystem Technical Committees Meeting, October 5, 2016. http://www.asmfc.org/files/Meetings/2016AnnualMeeting/HorseshoeCrabBoardSupplemental.pdf.
5. ASMFC Report to the Delaware Bay Ecosystem Technical Committee (DBETC) by the ARM Subcommittee October 2022 https://asmfc.org/uploads/file/63d2ee67HSC_ARM_Reports_Oct2022.pdf
6. Eisner C. Coastal biomedical labs are bleeding more horseshoe crabs with little accountability. NPR June 10, 2023 https://www.npr.org/2023/06/10/1180761446/coastal-biomedical-labs-are-bleeding-more-horseshoe-crabs-with-little-accountabi
7. 2016 Review of the Atlantic States Marine Fisheries Commission Fishery Management Plan for Horseshoe Crab (Limulus polyphemus) 2015 Fishing Year, http://www.asmfc.org/uploads/file/58b70d1eHORSESHOE CRABS_FMPReview_2016.pdf,
8. Hurton L, Berkson J. Potential causers of mortality for horseshoe crabs (Limulus polyphemus) during the biomedical bleeding process. Fish. Bull. 2006; 104:293–298. https://vtechworks.lib.vt.edu/bitstream/handle/10919/48012/hurton.pdf?sequence=1&isAllowed=y.
9. Interstate Fishery Management Plan for Horseshoe Crab, Atlantic States Marine Fisheries Commission Fisheries Commission. 1998. http://www.asmfc.org/uploads/file/hscFMP.pdf.
10. Lovenko C Demand for horseshoe crab blood for vaccine and drug testing has contributed to population declines. The Verge December 17, 2021 https://www.theverge.com/2021/12/17/22840263/horseshoe-crab-blood-medical-industry-controversy
11. Perry D. Massachusetts 2021 Compliance Report to the Atlantic States Marine Fisheries Commission – Horseshoe Crab. MA HSC compliance report 2021 public.pdf (mass.gov)
12. Smith, D.R., J.J. Newhard, C.P. McGowan, and C.A. Butler. 2020. The long-term effect of bleeding for Limulus Amebocyte Lysate on annual survival and recapture of tagged horseshoe crabs. Frontiers in Marine Science 7:607668.

Use of the Andrew+ Robot and OneLab Automated Liquid Handling Platform for Bacterial Endotoxin Testing

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Goal

To demonstrate that the Andrew+ and OneLab can be configured to execute the Pyrochrome® kinetic chromogenic assay.

Background

Pyrogen testing is a requirement for any injectable drug or medical device to ensure safety.[1] For example any vaccine or biologic must be tested for the presence of endotoxin in the final formulation. This can be accomplished by performing an endotoxin detection assay such as Pyrochrome®.[2] This Limulus Amebocyte Lysate (LAL) based assay[3,4] is an extremely sensitive enzymatic cascade method for the detection of sub nanogram quantities (10^{-9} g/mL) of endotoxin. The 96 well microplate based chromogenic LAL assay requires a fairly complex process necessitating numerous pipetting steps. Standard curve preparation, positive product control (PPC) preparation, sample arraying, and reagent dispensing are time consuming and tedious, but are critical steps in successful use of these assays. Automation of the liquid handling steps used in standard curve, PPC and sample preparation along with assay placement on the plate would significantly increase the efficiency for these assays. The automated process would liberate the analysts from repetitive time-consuming operations, lead to increased productivity, better quality in analytical work, and consistency in execution. In addition the added benefit of the OneLab event log would ensure a fully auditable process simplifying any future analyses and investigations.

LAL endotoxin assay execution note: As mentioned above these assays detect minute quantities of endotoxin in down to the 0.005EU/mL (EU: endotoxin units) range (i.e. sub nanogram (10^{-9} g)/mL). As indicated in the IFU all materials used for assay execution must be tested for potential interferences. Excellent lab technique and a clean environment are required to execute these assays. To address these requirements these results were collected with the Andrew+ in a static enclosure.

Here we present example results from the execution of the basic Pyrochrome® protocol with Andrew+ and OneLab utilizing the standard pipettes and dominos. Dominos are modular holders for tubes, plates, tips, reagents and other materials that can be configured in various combinations on the deck. Figure 1 shows the physical set up of the robot and the deck with the associated dominos.

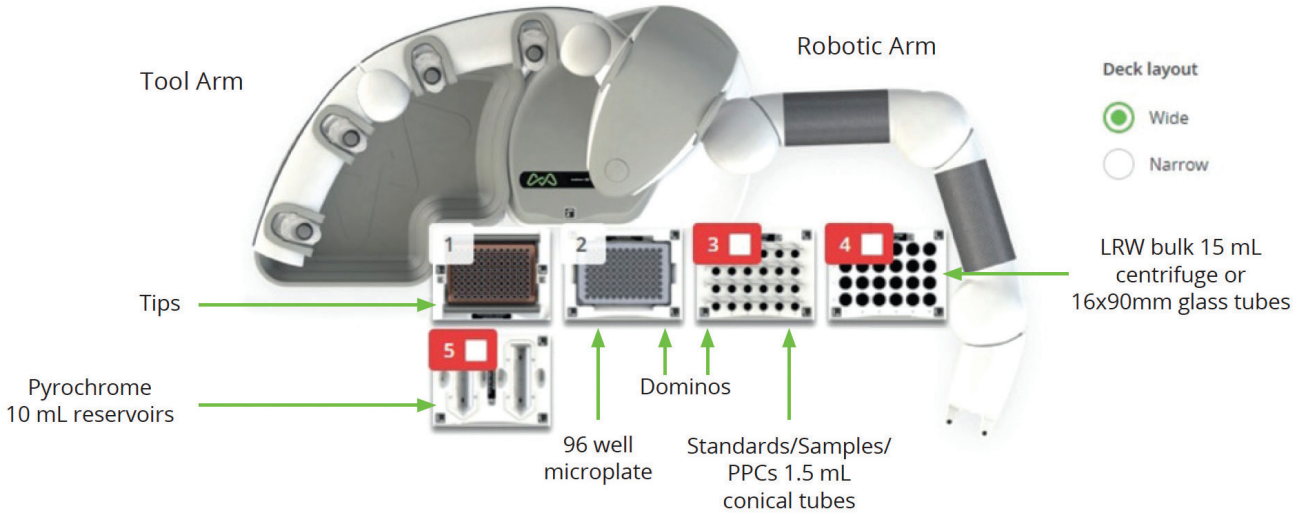


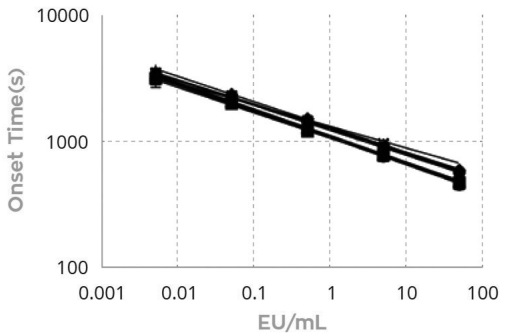
Figure 1. Andrew+ Confi guration. *Configuration used to execute the experiments for the Pyrochrome® results described in this technical note. The electronic Bluetooth connected pipettes used were the 300 µL multichannel and 300 µL single channel pipette. LRW is LAL reagent water.*

Results

This initial evaluation of the Andrew+ system addressed the creation of an OneLab protocol to execute a basic Pyrochrome® protocol using currently available dominos. The protocol was designed to evaluate the capability of the Andrew+ and OneLab to consistently create a standard curve and execute sample and PPC preparation and was conducted from multiple runs over four days.

A key element of the Pyrochrome® assay is the construction of the standard curve. For each plate a new standard curve was produced. These dilutions were created as serial dilutions 30 µL:270 µL starting with a 500 EU/mL stock to create a five point standard curve with concentrations of 50, 5, 0.5, 0.05 and 0.005 EU/mL. The standards were loaded onto each plate in triplicate. The standard curve onset time data (time for each sample to cross the 0.03 OD threshold) for each plate were analyzed by creating a log-log plot and performing a linear regression on the transformed data producing a slope, intercept, and R-value for each of the fourteen runs. These results are summarized in Figure 2. The variance for slopes and intercepts across the fourteen runs was 4.1% and 1.2%, respectively. The R-values were all excellent with the lowest one being 0.995 (the requirement is R≥ 0.980). These results are all within criteria for execution of a Pyrochrome® assay as indicted in the IFU.

In addition to the standard curve evaluation, a sample and two PPCs were included in these experiments. The sample was made manually in water to be ~ 0.5 EU/mL and loaded onto the deck. The sample and the 5 EU/mL standard were combined (10x dilution as with standard curve construction, 30 µL:270 µL) creating the two PPCs with Andrew+. The PPCs were expected to be about 1.0 EU/mL. The sample



	Slope	Intercept
Number of values	14	14
Minimum	-0.214	3.02
Maximum	-0.186	3.11
Range	0.0280	0.0900
Mean	-0.203	3.06
Std Deviation	0.00836	0.0359
Std. Error of Mean	0.00224	0.00958
Coefficient of variation	4.11%	1.17%

Figure 2.

and the PPCs were each loaded into ten wells on each plate. The data from across fourteen plates were examined and is summarized in Figure 3. In this case the average calculated values from each plate for each sample and PPC are presented. The calculated variances are larger for the samples (24.6%) and PPCs (16.3%) partly due to

	Sample EU/mL	PPC EU/mL	PPC Recovery
Number of values	14	14	14
Minimum	0.320	0.744	79.9
Maximum	0.787	1.34	127
Range	0.467	0.598	47.0
Mean	0.570	1.04	93.5
Std Deviation	0.141	0.169	13.0
Std. Error of Mean	0.0376	0.0451	3.48
Coefficient of variation	24.6%	16.3%	13.9%

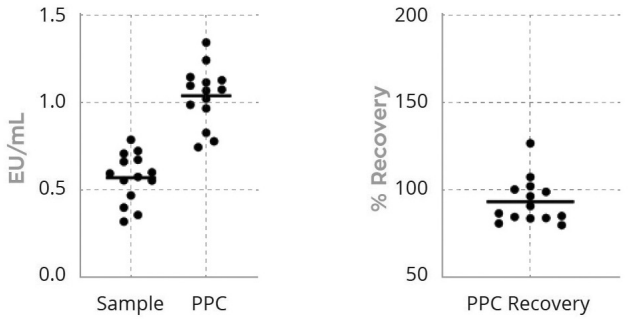


Figure 3.

the back calculation from the log- log plots. In addition, it should be noted that the samples were made manually for each day. The actual calculated mean for the samples across all plates was 0.57 EU/mL. This was within 14% of expectations across all 14 plates. The PPC of 1.04 EU/mL was within 4% of expectations across 14 plates. The average percent recovery for the PPC across all plates was 93.5%, well within the specified requirements of 50 to 200%.

Note: all data collected in the experiments presented here used the same lots of reagent and materials. Endotoxin samples were all derived from the same lot of reference standard endotoxin.

Summary

As presented above the results for the slopes and intercepts derived from fourteen plates have low variance and the R-values for the linear fits at 0.995 or better in each case. These results are consistent with the range of variances observed based on the historical data for the manual execution of this assay.

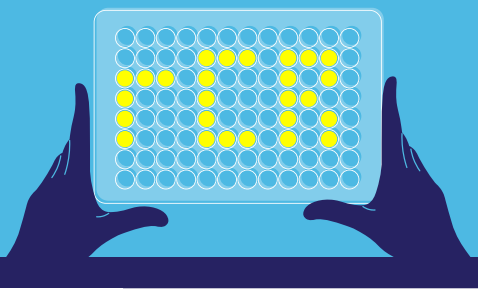
The sample variance was ~24.6% across the fourteen plates and the overall average result was within 14% of expectations. All PPCs were within the specified ranges of 50 to 200% consistent with the assay requirements and the average value across all plates was within 4% of expectations. Over all these results demonstrate than Andrew+ can execute the Pyrochrome® assay within the expected requirements outlined in the instructions for use.

There have been a number of publications describing custom automation processes for the execution of similar LAL based endotoxin testing methods.[5,6] While all have to overcome the same issues regarding consistency and sensitivity to contamination, none have the simplicity and accessibility demonstrated using Andrew+.

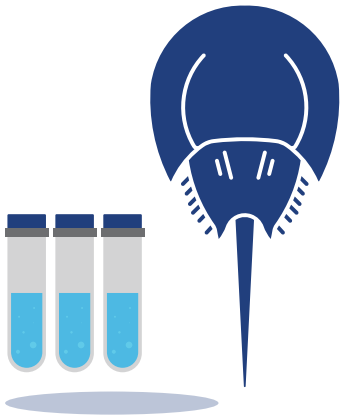
References

1. Bacterial Endotoxins Test, United States Pharmacopoeia <85>.
2. Pyrochrome® Instructions for use https://www.acciusa.com/tools-and-resources/package-insert-sheets/
3. Lindsay, G. K., P. F. Roslansky, and T. Novitsky. 1989. Single-Step, Chromogenic Limulus Amebocyte Lysate Assay for Endotoxin J. Clinic. Microbiol. 27:947-951.
4. Prior, R.B., 1990. The Limulus amoebocyte lysate test. In Clinical Applications of the Limulus Amoebocyte Lysate Test (p. 27). CRC Press Boca Raton, FL.
5. Tsuji KI, Martin PA, Bussey DM. 1984 Automation of chromogenic substrate Limulus amebocyte lysate assay method for endotoxin by robotic system. Applied and environmental microbiology. Sep 1;48(3):550-5.
6. Jorgensen, J.H. and Alexander, G.A., 1981. Automation of the Limulus amoebocyte lysate test by using the Abbott MS-2 microbiology system. Applied and environmental microbiology, 41(6):1316-1320.

The Future of Bacterial Endotoxin Testing



Conventional LAL Reagents vs Recombinant BET Technology



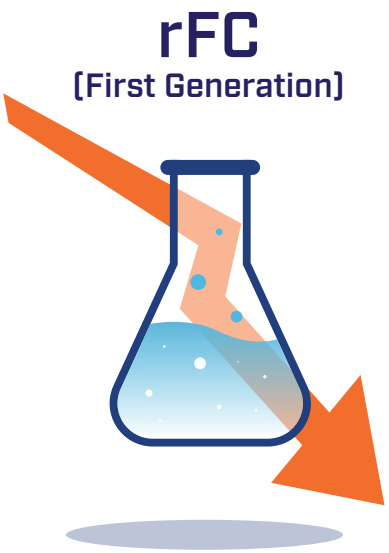
Accepted Test Methods

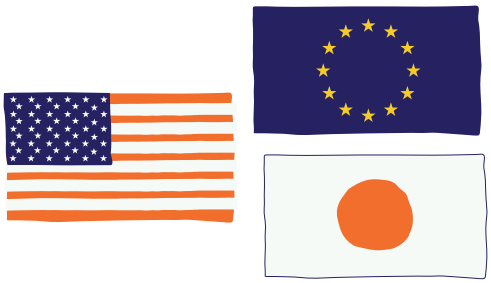
BET methods using traditional LAL reagents, will continue to have a very important place in the bacterial endotoxin testing space. They are, currently, the only FDA licensed reagents for BET.

Emerging Test Methods

The use of Recombinant Factor C (rFC) reagent as an alternative to LAL reagents (which are derived from the horseshoe crabs) has been implemented by some users, however, failed to achieve a wider industry's adoption over the 20 year period since its introduction.

A new recombinant alternative – Recombinant Cascade Reagent (rCR) was recently introduced providing the sustainable benefits of rFC reagents with none of the drawbacks of the rFC methods (refer to the table below for more information). Many early adopters have already implemented rCR for in house BET testing, especially for in process water samples.





Regulatory Outlook

In order for regulatory bodies such as the USP, EP & JP to incorporate recombinant based reagents for BET fully into their technical standards, it is expected to be documented that they perform equally to or better than the traditional LAL reagents. This is also referred to as comparability studies.

The Path Forward

Technology providers, subject matter experts and pharmaceutical companies have been working on comparability studies for several years. These industry stakeholders are committed to providing scientific evidence of whether the recombinant reagents for BET are equal to or better than the LAL reagents. With a growing body of evidence and a significant increase in adopting sustainable alternatives within the life science industry, the end users are becoming more confident in using recombinant reagents as an alternative to LAL reagents in their effort to convert to sustainably produced reagents. For your reference, the table below highlights the benefits of the rCR reagent compared to the conventional LAL reagent and the rFC reagent.

ATTRIBUTES EXPECTED FROM **BET** REAGENTS	TRADITIONAL **LAL** REAGENT	rFC REAGENT (FIRST GENERATION)	rCR REAGENT (SECOND GENERATION)
Data collection: Kinetic Assay?	✓	✗ Endpoint only	✓
Assay Setup: single step reconstitution?	✓	✗ rFC requires three reagents in a 1:4:5 ratio and a 10 min. pre-incubation step	✓
Instrumentation: Standard Absorbance Readers?	✓	✗ Fluorescent reader required	✓
Sourcing: Derived From Limulus polyphemus (e.g. LAL)?	✓	✗ Recombinantly prepared from *Carcinoscorpius or Tachypleus* Amebocyte Lysate (CAL/TAL)	✓ rCR is recombinant LAL
Multi-step Cascade Pathway Mechanism?	✓	✗	✓
Endotoxin Specific?	✗	✓	✓
Sustainable Reagent (animal free)?	✗	✓	✓

Report: A Demonstration of the Validation Process for Alternative Endotoxin Testing Methods Using PyroSmart NextGen® Recombinant Cascade Reagent

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[2]LAL Research, Central Research Laboratory, Seikagaku Corporation

The Bacterial Endotoxins Test (BET) uses Limulus amoebocyte lysate (LAL) reagents derived from the blood of horseshoe crabs for detection and quantification of endotoxins from Gram-negative bacteria in par enteral drug products and medical devices. Two types of recombinant reagents using genes cloned from the horseshoe crab genome have become available from several suppliers. One is a recombinant Factor C reagent (rFC), containing only recombinant Factor C, and the second is a recombinant Cascade Reagent (rCR) contain ing recombinant factor C, recombinant factor B and recombinant proclotting enzyme. Implementation of these recombinant reagents for BET requires validation that demonstrates results equal to or better than those deter mined by LAL reagents. Previous studies have shown that the PyroSmart NextGen® rCR meets the analytical performance requirements for both the plate and tube reader testing methods and provides equivalent results when testing samples containing autochthonous endotoxin. This study directly compares PyroSmart NextGen® to LAL reagent performance when testing a parenteral drug, which is a critical step for end-user implementa tion of alternative methods. It is the first published demonstration of an approach to the validation of alterna tive reagents that includes testing of a specific parenteral drug sample, and the data indicates that PyroSmart NextGen® is more precise when compared to LAL reagents. Relative recovery, linear regression, and Bland-Alt man plot analyses also illustrate that PyroSmart NextGen® results are equal to or better than those determined by naturally sourced LAL reagents. This indicates that PyroSmart NextGen® is a useful alternative method for quantifying bacterial endotoxins in parenteral drugs.

Key words: recombinant cascade reagent, equivalency, bacterial endotoxins test, lysate reagent

Introduction

In 1977, the United States Food and Drug Administration (FDA) announced the licensing of Limulus amoebocyte lysate (LAL) reagents derived from the hemolymph of living horseshoe crabs for the detection of potentially fatal endotoxins in biological products and medical devices. Over time, LAL became a compendial reagent for the Bacterial Endotoxins Test (BET) in the United States Pharmacopeia (USP), the European Pharmacopeia (Ph. Eur.) and the Japanese Pharmacopeia (JP).[1,2] More recently, the horseshoe crab genome has

been used to produce recombinant zymogen proteases, which are key components of recombinant Factor C reagents (rFC) and recombinant cascade reagents (rCR) such as PyroSmart NextGen®. These alternative reagents have the advantages of eliminating lot-to-lot variability and the false positives caused by triggering of the (1→3)-ß-D-glucan coagulation pathway which can occur when using naturally sourced LAL rea gents.1) Additionally, recombinant reagents promote sustaina ble endotoxin testing by meeting the replacement, reduction, and refinement principles for animal welfare.[3,4]

In the United States, recombinant reagents are considered alternative to compendial LAL reagents, therefore their use requires full method validation with results equal to or better than compendial LAL methods.[5,6] The Ph. Eur. has introduced a chapter on testing for bacterial endotoxins using rFC, although product-specific validation including demonstration of equivalency is still required to be performed by the end user.[7-9] To comply with all applicable regulations, the analyti cal performance of PyroSmart NextGen® using both the plate and tube reader testing methods has been validated, which allows for comprehensive equivalency analysis with LAL reagents. A previous study testing samples containing autochtho nous endotoxin established the methods for evaluating this equivalency against in-house criteria.[10,11]

This study assesses the analytical performance of PyroSmart NextGen®, Pyrochrome® (chromogenic LAL), and Pyrotell®-T (turbidimetric LAL) according to in-house acceptance criteria and directly compares the results to demonstrate equivalency. Analytical performance includes analysis of linearity, accuracy, precision, range, quantitation limit, and speci ficity according to USP <1225> and the ICH Q2 guideline.[12,13] Three lots of a parenteral drug sample were also tested using PyroSmart NextGen® and both LAL reagents for direct comparison of method suitability results according to USP <1085> and USP <85>.[14,15] This approach aligns with case study examples outlined in a recent FDA presentation. It specifies that non-product-specific analytical performance of alternative reagents should be evaluated by the manufacturer and the results when testing three lots of a specific product should be assessed for precision and accuracy by the end-user. This test ing should be performed using three lots of the alternative rea gent and three lots of a reference LAL reagent to demonstrate results equal to or better than LAL.[16] For this study, the prod uct-specific data constitutes a demonstration of method suit ability and was assessed for linearity, accuracy, precision, range, quantitation limit, specificity, and robustness accord ing to USP <1085>, USP <85>, USP <1225> and the ICH Q2 guideline (quantitative test for impurities).[12-15] Addition al comparison analysis was performed according to previous ly established methods to further illustrate the non-inferiority of PyroSmart NextGen®.[11] This is the first direct equivalency analysis of rCR and LAL reagent data, which demonstrates a non-product-specific method validation combined with prod uct-specific method suitability. This study can be used as a general example for end-users implementing alternative rea gents for BET if the assay details are optimized for specific needs.

Materials and Methods

Endotoxin - USP Reference Standard Endotoxin (USP RSE) was purchased from the United States Pharmaceutical Convention (MD, USA).

Sodium Citrate for Injection - Three lots of the paren teral drug Sodium Citrate for Injection were obtained from Seikagaku Corporation (Tokyo, Japan).

LAL Reagents - Three lots of Pyrochrome®, three lots of Pyrotell®-T, and one lot of Glucashield® buffer were obtained from Associates of Cape Cod, Inc. (MA, USA).

Recombinant Reagent - Three lots of PyroSmart Next Gen® were obtained from Associates of Cape Cod, Inc. (MA, USA).

Endotoxin Assays - Endotoxin was quantified using PyroSmart NextGen® and both LAL reagents according to their respective Instructions for Use. The onset time assay mode was used to measure the time required to reach a specific optical density threshold. The endotoxin concentration in samples was determined using the standard curve, which was constructed by plotting the log-converted onset time (Y-axis) against the log-converted standard concentration (X-axis).

Analytical Performance of PyroSmart NextGen® (Non-Product-Specific Method Validation Testing) - Three lots of PyroSmart NextGen® were used by three different ana lysts to perform a total of six plate reader assays and six tube reader assays over multiple days. The assays included a ten fold standard curve series of USP-RSE specific to each meth od (10, 1, 0.1, 0.01 EU/mL for the plate reader and 1, 0.1, 0.01, 0.001 EU/mL for the tube reader) tested in triplicate. The linearity, accuracy, precision, range, and quantitation lim it aspects of the analytical performance of PyroSmart Next Gen® were evaluated using these standard curve concentra tions according to USP <1225> and the ICH Q2 guideline.[12,13] Three separately prepared USP-RSE concentrations with high, medium, and low levels of endotoxin were incorporated (5, 0.5, 0.05 EU/mL for the plate reader and 0.3, 0.03, 0.003 EU/mL for the tube reader) and assessed for accuracy and precision. Although specificity for detecting endotoxin in the presence of (1→3)-ß-D-glucan was evaluated in previous studies not shown here, additional analysis when testing one lot of Sodium Citrate for Injection was included to provide an example using a sample matrix other than water.[10] Based on previous data, this testing was performed using the maximum valid dilution (MVD), the MVD/2, and the MVD/5 with corresponding positive product controls (PPCs) at a final concentra tion in the middle of each standard curve. These sample results were evaluated for suitable precision (repeatability), valid PPC recoveries, and

detectable endotoxin. The ICH guideline M10 and previous studies were referenced for all acceptance crite ria.[10,11,17]

Method Suitability of PyroSmart NextGen® (Product Specific Testing) - Three lots of the parenteral drug Sodium Citrate for Injection were tested at the previously-determined MVD/5 dilution with PPCs equivalent to the high, medium and low USP-RSE concentrations for the plate and tube read er testing methods. These results were assessed for linearity, accuracy, precision (repeatability), range, quantitation limit, specificity, and robustness to establish product-specific method suitability according to USP <1085>, USP <85>, USP <1225> and the ICH Q2 guideline (quantitative test for impurities).[12-15]

Equivalency Testing of PyroSmart NextGen® and LAL Reagents - The same assay setup (standard curve, sample dilutions, and USP-RSE concentrations) for PyroSmart Next Gen® was tested using Pyrochrome® in a plate reader and Pyrotell®-T in a tube reader. Both LAL reagents were recon stituted with glucan-blocking buffer to eliminate the possi bility of false positives caused by (1→3)-ß-D-glucan con tamination. Using in-house acceptance criteria, the analytical performance and method suitability results of each LAL rea gent were compared to those of PyroSmart NextGen® to dem onstrate equivalency.[10,11] The coefficient of variation results of the two methods were then compared utilizing the standard curve concentrations, the three separate USP-RSE concentra tions, and the samples PPCs to further demonstrate precision equivalency. Additional analysis was performed according to the methods and criteria described in a previous study.[11] The "relative recovery" of each USP-RSE concentration and all Sodium Citrate for Injection sample PPCs were evaluated by calculating the sample endotoxin concentration determined by PyroSmart NextGen® as a percentage of the endotoxin detect ed in the same sample determined by an LAL reagent.[18] Linear regression analysis comparing the endotoxin concentration in the sample PPCs and the three separate USP-RSE concen trations results determined by PyroSmart NextGen® on the Y-axis and the LAL reagent on the X-axis was included in the equivalency analysis. Bland-Altman plots of the same data were generated to illustrate any significant differences between PyroSmart NextGen® and the LAL reagents.[10,19]

Results

Analytical Performance of PyroSmart NextGen® (Non Product-Specific Method Validation Testing) - All standard curve concentrations and the three separately prepared USP RSE concentrations quantified using PyroSmart NextGen® meet the linearity, accuracy, precision, range, and quantitation limit specifications (Tables 1 and 2). Therefore, PyroSmart NextGen® analytical performance as defined by USP <1225> and the ICH Q2 guideline has been demonstrated.[12,13] Addi tional analysis of specificity when testing a Sodium Citrate for Injection sample matrix also meets the precision, accuracy, and endotoxin detection criteria.

Method Suitability of PyroSmart NextGen® (Product Specific Testing) - The three lots of Sodium Citrate for Injec tion tested with three PPC concentrations satisfy the lineari ty, accuracy, precision, range, quantitation limit, specificity, and robustness criteria (Tables 3 and 4). According to USP <1085>, USP <85>, USP <1225> and the ICH Q2 guideline (quantitative test for impurities), product-specific method suitability of PyroSmart NextGen® has been demonstrated.[12-15]

Equivalency Testing of PyroSmart NextGen® and LAL Reagents - Both the Pyrochrome® and Pyrotell®-T LAL rea gents produce results that meet the in-house acceptance crite ria for analytical performance and method suitability. Direct comparison of the LAL reagents to PyroSmart NextGen® illus trates that PyroSmart NextGen® has equal or better results for all parameters (Tables 1-4). Additional precision analysis dem onstrates that the maximum coefficient of variation seen with PyroSmart NextGen® is 20.89% for plate and tube reader test ing, whereas Pyrochrome® has a maximum of 23.22% and Pyrotell®-T has a maximum of 29.24% (Figure 1). All samples containing detectable (in this case, added) endotoxin (three separately prepared USP-RSE concentrations and Sodium Cit rate for Injection sample PPCs) have relative recovery results within 50-200% (Figure 2). Before linear regression and Bland Altman plot analysis, a normality test was completed, which determined that the three separately prepared USP-RSE con entration and sample PPC data should be transformed loga rithmically. The subsequent linear regression analysis of the plate reader data results in slope of 0.9532 and a 95% confi dence interval (CI) of 0.9418 to 0.9645. The tube reader linear regression has a slope

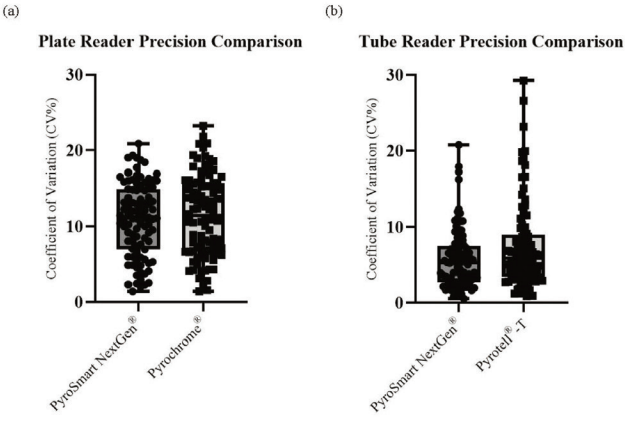

Figure 1. (a) Precision Comparison of the Endotoxin Concentrations Determined by PyroSmart NextGen® and Pyrochrome® Using a Plate Reader. (b) Precision Comparison of the Endotoxin Concentrations Determined by PyroSmart NextGen® and Pyrotell®-T Using a Tube Reader.

All results with calculable coefficient of variation values were included (the standard curve, the three separately prepared USP-RSE concentrations, and the sample PPCs).

Table 1. Assessment of PyroSmart NextGen® Analytical Performance Compared to Pyrochrome® According to USP <1225> and the ICH Q2 Guideline

Analytical Performance Characteristics		PyroSmart NextGen® Results	Pyrochrome®Results	Acceptance Criteria
1. Linearity (absolute value, correlation coefficient)	**0.01-10 EU/mL**	Minimum: 0.998 Maximum: 1.000	Minimum: 0.998 Maximum: 1.000	|r| ≥0.980
2. Accuracy (recovery)	**Standard Curve** 0.01 EU/mL 0.1 EU/mL 1.0 EU/mL 10 EU/mL **USP-RSE** 0.05 EU/mL 0.5 EU/mL 5.0 EU/mL	**Min-Max (%)** 85-98 99-121 109-122 85-95 **Min-Max (%)** 109-118 116-131 116-134	**Min-Max (%)** 88-100 98-116 105-116 87-98 **Min-Max (%)** 95-115 107-127 130-153	50-200%
3. Precision **3-1 Repeatability (CV)**	**Standard Curve** 0.01 EU/mL 0.1 EU/mL 1.0 EU/mL 10 EU/mL **USP-RSE** 0.05 EU/mL 0.5 EU/mL 5.0 EU/mL	**Min-Max (%)** 3-21 5-11 8-18 12-17 **Min-Max (%)** 6-16 5-15 6-12	**Min-Max (%)** 2-21 7-13 7-17 13-18 **Min-Max (%)** 6-19 4-17 4-11	CV ≤30%
3-2 Intermediate Precision (95% CI for CV)	**Standard Curve** 0.01 EU/mL 0.1 EU/mL 1.0 EU/mL 10 EU/mL **USP-RSE** 0.05 EU/mL 0.5 EU/mL 5.0 EU/mL	**Min-Max (%)** 11-19 7-13 10-17 10-17 **Min-Max (%)** 8-14 7-13 7-12	**Min-Max (%)** 9-15 7-13 9-16 11-20 **Min-Max (%)** 9-16 8-15 7-13	CV ≤30%
4. Range		**0.01-10 EU/mL**	**0.01-10 EU/mL**	Precision, accuracy, and linearity at suitable level
5. Quantitation Limit		**At 0.01 EU/mL** Accuracy: 85-98% Repeatability: 3-21%	**At 0.01 EU/mL** Accuracy: 88-100% Repeatability: 2-21%	The lowest concentration of endotoxin that can be quantitatively determined with suitable precision and accuracy
6. Specificity		**Lot 1 Samples** Sample Concentration: <5.56 - <1.11 EU/mL Repeatability: 0-8% PPC Recovery: 96-134%	**Lot 1 Samples** Sample Concentration: <5.56 - <1.11 EU/mL Repeatability: 0-11% PPC Recovery: 88-121%	For a sample matrix that does not contain endotoxin, the endotoxin concentration is determined as undetected with suitable precision and accuracy (PPC recovery)

Note: Specificity evaluated here is an additional example using a sample matrix other than water. Reproducibility (multiple locations) and specificity for detecting endotoxin in the presence of (1➞3)-ß-D-glucan were both analyzed in previous studies not included here, and the results met the acceptance criteria.[10]

Table 2. Assessment of PyroSmart NextGen® Analytical Performance Compared to Pyrotell®-T According to USP <1225> and the ICH Q2 Guideline

Analytical Performance Characteristics		PyroSmart NextGen® Results	Pyrotell®-T Results	Acceptance Criteria
1. Linearity (absolute value, correlation coefficient)	**0.01-10 EU/mL**	Minimum: 0.996 Maximum: 0.998	Minimum: 0.987 Maximum: 0.993	|r| ≥0.980
2. Accuracy (recovery)	**Standard Curve** 0.01 EU/mL 0.1 EU/mL 1.0 EU/mL 10 EU/mL **USP-RSE** 0.003 EU/mL 0.03 EU/mL 0.3 EU/mL	**Min-Max (%)** 83-87 110-117 121-132 79-84 **Min-Max (%)** 80-120 112-139 102-128	**Min-Max (%)** 63-75 134-171 136-146 67-74 **Min-Max (%)** 76-124 118-173 93-116	50-200%
3. Precision **3-1 Repeatability (CV)**	**Standard Curve** 0.001 EU/mL 0.01 EU/mL 0.1 EU/mL 1.0 EU/mL **USP-RSE** 0.003 EU/mL 0.03 EU/mL 0.3 EU/mL	**Min-Max (%)** 3-12 3-9 1-4 1-10 **Min-Max (%)** 1-7 3-10 2-6	**Min-Max (%)** 3-20 3-9 2-12 2-9 **Min-Max (%)** 3-29 2-15 3-12	CV ≤35% 0.001 EU/mL CV ≤30% 0.01-1.0 EU/mL
3-2 Intermediate Precision (95% CI for CV)	**Standard Curve** 0.001 EU/mL 0.01 EU/mL 0.1 EU/mL 1.0 EU/mL **USP-RSE** 0.003 EU/mL 0.03 EU/mL 0.3 EU/mL	**Min-Max (%)** 7-12 5-9 3-5 4-7 **Min-Max (%)** 12-22 8-15 7-12	**Min-Max (%)** 11-20 7-13 5-9 5-9 **Min-Max (%)** 17-30 14-25 8-15	CV ≤35% 0.001 EU/mL CV ≤30% 0.01-1.0 EU/mL
4. Range		**0.001-1.0 EU/mL**	**0.001-1.0 EU/mL**	Precision, accuracy, and linearity at suitable level
5. Quantitation Limit		**At 0.001 EU/mL** Accuracy: 83-87% Repeatability: 3-12%	**At 0.001 EU/mL** Accuracy: 63-75% Repeatability: 3-20%	The lowest concentration of endotoxin that can be quantitatively determined with suitable precision and accuracy
6. Specificity		**Lot 1 Samples** Sample Concentration: <5.56 - <1.11 EU/mL Repeatability: 0-9% PPC Recovery: 116-140%	**Lot 1 Samples** Sample Concentration: <5.56 - <1.11 EU/mL Repeatability: 0-19% PPC Recovery: 121-168%	For a sample matrix that does not contain endotoxin, the endotoxin concentration is determined as undetected with suitable precision and accuracy (PPC recovery)

Note: Specificity evaluated here is an additional example using a sample matrix other than water. Reproducibility (multiple locations) and specificity for detecting endotoxin in the presence of (1➞3)-ß-D-glucan were both analyzed in previous studies not included here, and the results met the acceptance criteria.[10,11]

Table 3. Assessment of PyroSmart NextGen® Method Suitability Compared to Pyrochrome® According to USP <1085>, USP <85>, USP <1225> and the ICH Q2 Guideline (quantitative test for impurities)

Method Suitability Characteristics		PyroSmart NextGen® Results	Pyrochrome®Results	Acceptance Criteria
1. Linearity (absolute value, correlation coefficient)	**0.05-5.0 EU/mL**	0.990	0.994	|r| ≥0.980
2. Accuracy (PPC recovery)	**Spiked Sample** 0.05 EU/mL 0.5 EU/mL 5.0 EU/mL	**Min-Max (%)** 91-138 122-177 88-134	**Min-Max (%)** 90-122 127-186 115-152	50-200%
3. Precision **3-1 Repeatability (CV)**	**Spiked Sample** 0.05 EU/mL 0.5 EU/mL 5.0 EU/mL	**Min-Max (%)** 1-12 7-17 11-19	**Min-Max (%)** 1-9 7-18 10-23	CV ≤30%
4. Range		**0.05-5.0 EU/mL**	**0.05-5.0 EU/mL**	Precision, accuracy, and linearity at suitable level
5. Quantitation Limit		**At 0.05 EU/mL** Accuracy: 91-138% Repeatability: 1-12%	**At 0.01 EU/mL** Accuracy: 90-122% Repeatability: 1-9%	The lowest concentration of endotoxin that can be quantitatively determined with suitable precision and accuracy
6. Specificity		**Lot 1-3 Samples** Sample Concentration: <1.11 EU/mL Repeatability: 1-19% PPC Recovery: 88-177%	**Lot 1-3 Samples** Sample Concentration: <1.11 EU/mL Repeatability: 0-1-23% PPC Recovery: 90-186%	For a sample matrix that does not contain endotoxin, the endotoxin concentration is determined as undetected with suitable precision and accuracy (PPC recovery)
7. Robustness **7-1 Intermediate Precision (95% CI for CV)**	**Spiked Sample** 0.05 EU/mL 0.5 EU/mL 5.0 EU/mL	**Min-Max (%)** 9-13 13-18 15-20	**Min-Max (%)** 8-11 13-18 15-20	CV ≤30%
Note: The pH of the mixture of the reagent and sample solution was determined to be between 6.0 and 8.0.				

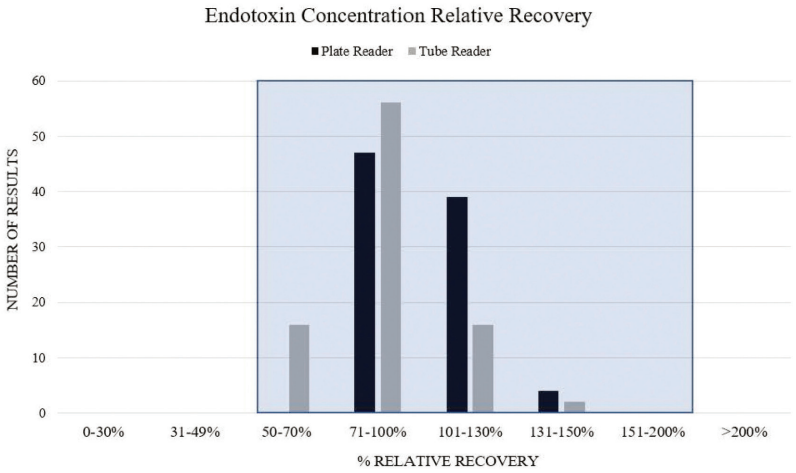

Figure 2. Relative Recovery Analysis Summary for the Endotoxin Concentration in Sample PPCs and the Three Separately Prepared USP-RSE Concentra tions Determined Using the Plate and Tube Reader Methods.

Each column in the graph represents the number of results that have relative recoveries within each defined range. The shaded box highlights all results within 50-200%.

of 1.023 and a 95% CI of 1.000 to 1.046 (Figure 3). The Bland-Altman plate reader testing bias is -2.899 and 97% of data points are within the 95% upper and lower limits of agreement (LOA) whereas the tube reader has a bias of 5.301 and the same percentage of samples (97%) within the LOA (Figure 4).

Discussion

The USP *General Notices and Requirements* 6.30 and the 2012 FDA *Guidance for Industry: Pyrogen and Endotoxins Testing* state that alternative reagents must be validated with results equivalent to or better than the results of LAL rea gents.[4,5] A previous study has

Table 4. Assessment of PyroSmart NextGen® Method Suitability Compared to Pyrotell®-T According to USP <1085>, USP <85>, USP <1225> and the ICH Q2 Guideline (quantitative test for impurities))

Method Suitability Characteristics		PyroSmart NextGen® Results	Pyrotell®-T Results	Acceptance Criteria
1. Linearity (absolute value, correlation coefficient)	**0.03-3.0 EU/mL**	0.994	0.996	|r| ≥0.980
2. Accuracy (PPC recovery)	**Spiked Sample** 0.003 EU/mL 0.03 EU/mL 0.3 EU/mL	**Min-Max (%)** 82-128 107-171 94-129	**Min-Max (%)** 97-168 158-199 110-133	50-200%
3. Precision **3-1 Repeatability (CV)**	**Spiked Sample** 0.003 EU/mL 0.03 EU/mL 0.3 EU/mL	**Min-Max (%)** 3-21 2-17 1-11	**Min-Max (%)** 1-27 1-17 3-14	CV ≤30%
4. Range		**0.003-0.3 EU/mL**	**0.003-0.3 EU/mL**	Precision, accuracy, and linearity at suitable level
5. Quantitation Limit		**At 0.003 EU/mL** Accuracy: 82-128% Repeatability: 3-21%	**At 0.01 EU/mL** Accuracy: 97-168% Repeatability: 1-27%	The lowest concentration of endotoxin that can be quantitatively determined with suitable precision and accuracy
6. Specificity		**Lot 1-3 Samples** Sample Concentration: <1.11 EU/mL Repeatability: 1-21% PPC Recovery: 82-171%	**Lot 1-3 Samples** Sample Concentration: <1.11 EU/mL Repeatability: 1-27% PPC Recovery: 97-199%	For a sample matrix that does not contain endotoxin, the endotoxin concentration is determined as undetected with suitable precision and accuracy (PPC recovery)
7. Robustness **7-1 Intermediate Precision (95% CI for CV)**	**Spiked Sample** 0.003 EU/mL 0.03 EU/mL 0.3 EU/mL	**Min-Max (%)** 13-18 14-19 8-11	**Min-Max (%)** 13-18 7-10 7-10	CV ≤30%

Note: The pH of the mixture of the reagent and sample solution was determined to be between 6.0 and 8.0.

validated the analytical perfor mance of PyroSmart NextGen® when using the plate read er in both the rate and onset assay modes. The capability of PyroSmart NextGen® to determine the potency of different bacterial strains, and to recover endotoxin from various inter fering pharmaceuticals at a level equivalent to LAL reagents was successfully demonstrated.[10] A second study validated PyroSmart NextGen® tube reader analytical performance and demonstrated result equivalency when testing water samples containing autochthonous endotoxin.[11] This study provides further confirmation of PyroSmart NextGen® analytical per formance in a plate and tube reader. It also demonstrates that the product-specific method suitability data meets the accura cy and precision requirements when testing three lots of Sodi um Citrate for Injection.[14,15,20] Additional analysis of linearity, range, quantitation limit, specificity, and robustness using the method suitability data was performed according to the USP <1225> and the ICH Q2 guideline quantitative test for impurities. The results meet all criteria and demonstrate the full analytical capability of PyroSmart NextGen® when test ing a monograph product (Sodium Citrate for Injection) for the first time.[12,13] In addition to evaluation of PyroSmart Next Gen®, the same testing was performed using LAL reagents, and for consistent analysis of equivalency all analytical per formance and method suitability data was assessed accord ing to the same in-house acceptance criteria. The precision results of the endotoxin concentrations determined by all rea gents were also compared. This is the first direct comparison of non-product specific and product-specific rCR test results to those obtained by LAL reagents. The approach used aligns with FDA case studies, which outline the validation of alterna tive reagents for BET within the framework of non-product specific analytical performance and product-specific testing to demonstrate equivalency to LAL.[16]

The non-inferiority of PyroSmart NextGen® is further supported in this study by additional equivalency assessment. Because there are not specific guidelines for comparison stud ies, the three separately prepared USP-RSE concentrations and samples containing added endotoxin were evaluated for equiv alency according to the methods and in-house criteria defined in the large-scale water sample study.

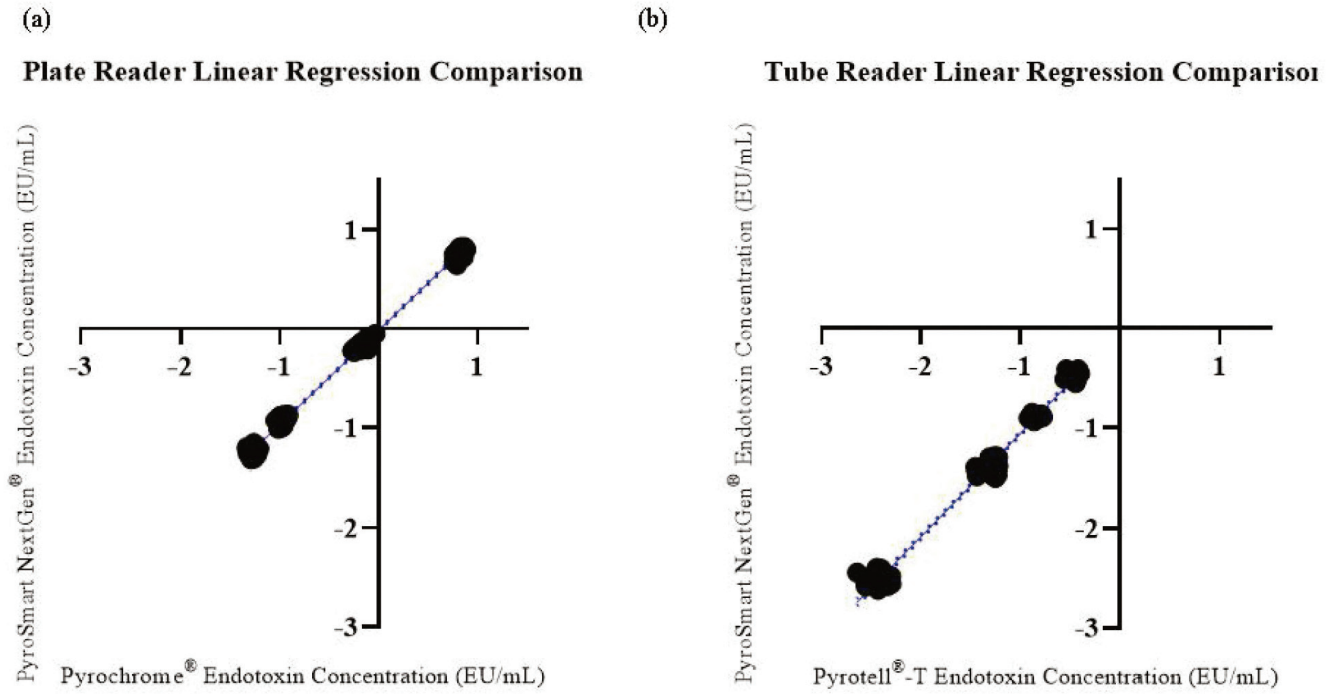

Figure 3. (a) Linear Regression Analysis of the Samples Containing Added Endotoxin and the Three Separately Prepared USP-RSE Concentrations Tested with Pyrochrome® Compared to Those Tested with PyroSmart NextGen®. (b) Linear Regression Analysis of the Samples Containing Added Endotoxin and the Three Separately Prepared USP-RSE Concentrations Tested with Pyrotell®-T Compared to Those Tested with PyroSmart NextGen®.

The solid line depicts the slope, and the dotted lines are the 95% confidence interval (CI) of the slope.

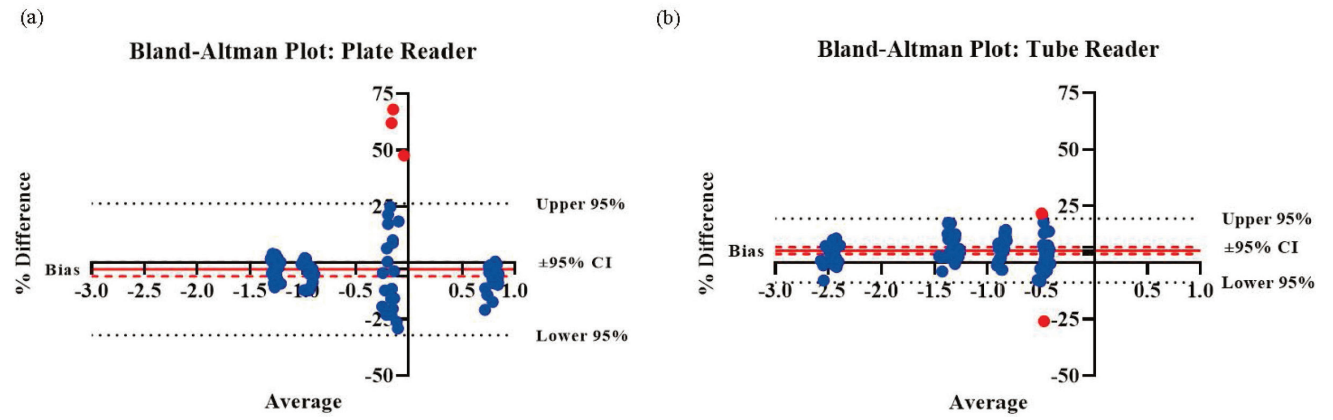

Figure 4. (a) Bland-Altman Plot Analysis of the Three Separately Prepared USP-RSE Concentrations and the Samples with Added Endotoxin Determined by PyroSmart NextGen® and by Pyrochrome® Tested Using a Plate Reader. (b) Bland-Altman Plot Analysis of the Three Separately Prepared USP-RSE Concen trations and the Samples with Added Endotoxin Determined by PyroSmart NextGen® and by Pyrotell®-T Tested Using a Tube Reader.

A bias (solid red line) of zero indicates that the results are identical, and the dotted red lines are the 95% confidence interval (CI) of the bias. The red data points are those out side of the 95% upper and lower limits of agreement (LOA, dotted black lines).

This includes calculating the relative recovery of the endotoxin concentration in samples for both the plate and tube reader methods with a criteri on that 70% of samples should have results within 50-200%. Comparison of PyroSmart NextGen® and LAL reagents in a plate and tube reader were also evaluated using linear regres sion and Bland-Altman plot analysis. The original water study criteria stated that the linear regression slope must be between 0.7 and 1.3, and the Bland-Altman plots must have at least 95% of data points within the LOA.[11] In this study, 100% of the endotoxin concentration results have relative recovery results within 50-200%. This greatly exceeds the original cri terion, likely because known concentrations of USP-RSE were added to the highly purified parenteral drug sample, which also presumably does not contain any contaminants that would affect the assay. Linear regression analysis resulted in slopes of 0.9532 and 1.023, which meet the pre-determined criterion, and the Bland-Altman plots resulted in 97% of the data within the 95% LOA for both plate and tube reader method compar isons. Various publications involving different types of sam ples further support that PyroSmart NextGen® is comparable to LAL and other recombinant reagents including rFC.[10,11,21-23]

As shown in this and two previous studies, PyroSmart NextGen® is a robust rCR that meets all analytical performance and method suitability criteria in both a plate and tube reader. Endotoxin concentration coefficient of variation analysis dem onstrates that PyroSmart NextGen® is more precise when com pared to LAL reagents. The development and manufacturing processes also meet the quality standards that are applied to FDA-licensed LAL reagents. The wide standard curve range and multiple test method options for PyroSmart NextGen® enable direct comparison to various LAL reagents, and opti mization by the end-user to suit their particular needs. Assay specifics such as dilution factors, standard curve range, add ed endotoxin concentrations and acceptance criteria must be optimized for each end-user's requirements. It is also impor tant to note that this study was designed in accordance with the current regulatory and guideline documents at the time of publication, and future updates may change the requirements. Further research using various parenteral drugs and multiple endotoxin detection systems could be used to support the use of alternative methods for BET. This, and two previous stud ies illustrate an approach that can be used for recombinant rea gent validation and implementation. They demonstrate that PyroSmart NextGen® meets all in-house criteria with results that are equivalent to or better than those determined by nat urally sourced LAL reagents when testing multiple sample types.

Conflict of interest - Kelley, Stevens, Akiyoshi are employ ees of Associates of Cape Cod, Inc. and Jahngen is a consult ant to Associates of Cape Cod, Inc., Oda is an employee of both Associates of Cape Cod, Inc. and Seikagaku Corporation.

References

1. Tindall B, Demircioglu D, Uhlig T. Recombinant bacterial endotoxin testing: a proven solution. Biotechniques, 70, 290–300 (2021).
2. Licensing of. Limulus amebocyte lysate, use as an alternative for rabbit pyrogen test. In: Federal Register. Washington, 42(213) (1977).
3. Interagency Coordinating Committee on the Validation of Alternative Methods Authorization Act of 2000. Public Law, 106–545 (2000).
4. European Union Directive 2010/63/EU, (2010).
5. United States Pharmacopeia. General Notices and Requirements. Sec tion 6.30.
6. United States Food and Drug Administration. Guidance for Industry. Pyrogen and Endotoxins Testing: Questions and Answers, (June 2012).
7. European Pharmacopeia. Chapter 2.6.32 Test for Bacterial Endotoxins Using Recombinant Factor C.
8. European Pharmacopeia. General Notices. Section 1.1.2.4.
9. European Pharmacopeia. Chapter 5.1.10 Guidelines for Using the Test for Bacterial Endotoxins.
10. Stevens I, Ogura N, Kelley M, D'Ordine RL, Mizumura H, Oda T, Akiyoshi J, Jahngen EG. Advanced recombinant cascade reagent PyroSmart NextGen® for bacterial endotoxins test as described in the pharmacopeias. BPB Reports, 5, 105–114 (2022).
11. Kelley M, Stevens I, Akiyoshi J, Jahngen EG. Evaluation of recom binant cascade reagent PyroSmart NextGen® and Limulus amebocyte lysate equivalency in a plate and tube reader for bacterial endotoxins testing. BPB Reports, 6, 11–15 (2023).
12. United States Pharmacopeia. Chapter <1225> Validation of Compendi al Procedures.
13. International conference of harmonization of technical requirements for registration of pharmaceuticals for human use. ICH harmonized tri partite guideline. Validation of Analytical Procedures: Text and Meth odology Q2 (R1), November 2005.
14. United States Pharmacopeia. Chapter <1085> Guidelines on the Endo toxins Test.
15. United States Pharmacopeia. Chapter <85> Bacterial Endotoxins Test.
16. Candau-Chacon R. FDA Perspective on Recombinant Endotoxin Detection Systems. [Webinar]. 2021 USP Virtual Open Forum: Alter natives to Compendial Reagents used in the Bacterial Endotoxins Text. United States Food and Drug Administration, (2021). https://www.usp. org/sites/default/files/usp/document/events-training/03-fda-perspec tive-on-recombinant-reyes-candau-chacon-final.pdf
17. International conference of harmonization of technical requirements for registration of pharmaceuticals for human use. ICH harmonized tri partite guideline. Bioanalytical Method Validation and Study Sample Analysis M10, July 2022.
18. Akers J, Guilfoyle DE, Hussong D, McCullough K, Mello R, Singer D, Tidswell E, Tirumalai R. Functional challenges for alternative bac terial endotoxins tests part 2: comparability. Am. Pharm. Rev., 23, 18– 27 (2020).
19. Giavarina D. Understanding Bland Altman analysis. Biochem. Med. (Zagreb), 25, 141–145 (2015).
20. United States Pharmacopeia. Chapter <1223> Validation of Alternative Microbiological Methods.
21. Muroi M, Ogura N, Mizumura H, Aketagawa J, Oda T, Tanamoto KI. Application of a Recombinant Three-Factor Chromogenic Reagent, PyroSmart, for Bacterial Endotoxins Test Filed in the Pharmacopeias. Biol. Pharm. Bull., 42, 2024–2037 (2019).
22. Kikuchi Y, Haishima Y, Fukui C, Murai T, Nakagawa Y, Ebisawa A, Matsumura K, Ouchi K, Oda T, Mukai M, Masuda T, Katto Y, Takas uga Y, Takaoka A. Collaborative Study on the Bacterial Endotox ins Test Using Recombinant Factor C-Based Procedure for Detection of Lipopolysaccharides (Part 1). Pharm. Med. Device Regul. Sci., 48, 252–260 (2017).
23. Kikuchi Y, Haishima Y, Fukui C, Nakagawa Y, Ebisawa A, Morioka T, Matsumura K, Ouchi K, Uchida K, Martinez O, Oda T, Mukai M, Masuda T, Tsukihashi Y, Takasuga Y, Takaoka A. Collaborative Study on the Bacterial Endotoxins Test Using Recombinant Factor C-Based Procedure for Detection of Lipopolysaccharides (Part 2). Pharm. Med. Device Regul. Sci., 49, 708–718 (2018).

It's Easy To See

BET Sustainability

LAL Reagent Comparison Table	Conventional LAL Reagent	ACC's PyroSmart NextGen® (rCR) Reagent	First Generation Competitor (rFC) Reagent
Sustainable Reagent (animal free)	**No**	✓ **Horseshoe Crab Blood Free**	✓ **Horseshoe Crab Blood Free**
Kinetic Assay	Kinetic	✓ Kinetic	✗ **No.** Endpoint only
Assay Setup	Single step reconstitution	✓ Single step reconstitution	✗ **No.** rFC requires three reagents in a 1:4:5 ratio and a 10 min. pre-incubation step
Same Standard Plate Reader	Incubating plate or tube reader at 405 nm	✓ **Yes.** Incubating plate or tube reader at 405 nm	✗ **No.** Fluorescent reader required
Derived From *Limulus* Amebocyte Lysate (LAL)	LAL	✓ **Yes.** rCR is recombinant LAL	✗ **No.** Based on *Carcinoscorpius* or *Tachypleus* Amebocyte Lysate (CAL/TAL)
Multi-step Cascade Pathway	Yes	✓ **Yes**	✗ **No**
Endotoxin Specific	No	✓ Endotoxin Specific	✓ Endotoxin Specific

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