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Beyond LAL: Next-Gen Recombinant Methods for Endotoxin Testing

Navigating the Technological Frontier of Contamination Detection in Drug Production

A Supplement to American Pharmaceutical Review

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IT'S OFFICIAL!

The Microbiology Expert Committee has approved endotoxin testing using non-animal derived reagents. Chapter <86> of the USP now includes recombinant cascade (rCR) methods like **PyroSmart NextGen®**.

First-Gen. Second-Gen. NEXT-GEN.

Wherever you are on your BET journey, we've got you covered.

BETransformed. ACC transformed endotoxin testing in 1974 with the introduction of its Pyrotell® lysate gel-clot reagent and then again with its chromogenic and turbidimetric tests, Pyrochrome® and Pyrotell®-T.

Now, we are transforming the industry again with **PyroSmart NextGen®**, a groundbreaking recombinant BET solution with all of the quality and consistency you have come to expect from our traditional LAL reagents.

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Learn more at **acciusa.com/BETransformed**.

PyroSmart®
NEXTGEN Recombinant Cascade Reagent (rCR)

A Note from the Editor

Mike Auerbach

Editor in Chief
American Pharmaceutical Review

The pharmaceutical industry is witnessing a significant shift towards more sustainable and ethical practices, particularly in endotoxin testing. This publication explores the growing trend of using recombinant cascade reagents (rCR) as an alternative to traditional Limulus Amebocyte Lysate (LAL) tests, reflecting the industry's commitment to environmental stewardship and animal welfare.

For decades, endotoxin testing has relied on LAL derived from horseshoe crab blood, raising concerns about the impact on these ancient creatures and the sustainability of the process. In response, scientists have developed recombinant alternatives that promise to deliver comparable results while addressing these ethical and environmental concerns.

Recombinant cascade reagents utilize the same enzymatic cascade as traditional LAL reagents but are produced through recombinant DNA technology. This approach not only eliminates the need for horseshoe crab blood but also offers enhanced specificity by removing the potential for cross-reactivity with 1,3-β-D-glucans.

As the pharmaceutical industry increasingly prioritizes sustainability, the adoption of rCR methods is gaining momentum. This supplement explores the science behind these recombinant reagents, their benefits, and the challenges and opportunities they present for manufacturers transitioning to more sustainable endotoxin testing processes.

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July 17, 2024

Accelerating the Transition to Sustainable Endotoxin Detection Solutions

To whom this may concern,

We are writing to you as an alliance dedicated to advancing sustainability and innovation in the bacterial endotoxin testing (BET) field. Our mission is to educate pharmaceutical and medical device companies on the benefits of initiating the transition from using animal-derived natural resources, such as *Limulus* or *Tachypleus* amebocyte lysates (LAL or TAL), to their recombinant alternatives for Bacterial Endotoxin Testing as part of quality control testing.

Together, we recognize the key impacts of adopting recombinant technologies for BET:

1. Mitigation of global supply chain risks and improved resilience of supply chain;
2. Significantly reducing the need for collecting horseshoe crabs to produce lysate is decreasing the environmental pressures on their ecosystems;
3. Implementation of innovative and sustainable technical advancements.

The adoption of recombinant solutions is supported by the positively evolving regulatory landscape making the transition to recombinant solutions even more accessible for pharmaceutical and medical device companies. Additionally, companies applying sustainable practices can benefit economically through financial incentives for specific environmentally positive and climate-related projects. This presents a unique opportunity for the industry to align quality control lab practices with sustainability measures while continuously ensuring regulatory compliance and product safety.

This subject has now taken the attention of the investment community. BNP Paribas Asset Management has recently publicly called for the switch from LAL/TAL to synthetic alternatives due to their concerns about the risks associated with dependency on horseshoe crabs. Moreover, growing numbers of investors have demonstrated their concern about corporate impacts and dependencies on nature by joining the investor-led initiative Nature Action 100, which has more than 200 investor participants representing over US$ 28 trillion in assets under management or advice. The biotechnology and pharmaceutical sector is one of eight systemically important sectors that the initiative has identified for initial investor engagement.

The undersigned companies believe that recombinant endotoxin tests are suitable in replacement of LAL testing after appropriate suitability and comparability testing are performed. We offer you the opportunity to explore available publications, research papers and other resources which compare recombinant methods to the traditional LAL methods. These resources will help you make informed decisions regarding the right recombinant technologies to fit your needs and the intended use. By staying informed and proactive, we can collectively accelerate this shift towards more sustainable and socially responsible processes.

Thank you for your attention to this matter.

With best regards,

ACC

Main manufacturing site: East Falmouth, Massachusetts, USA

Supply Worldwide through direct subsidiaries (Liverpool, UK and Amsterdam, Netherlands) or distributor network: Available at www.acciusa.com

Training materials: Technical eLearning modules, white paper, references:
Peer review publications: https://www.acciusa.com/tools-and-resources/educational-content/acc-rcr-reference-list

Full texts available upon request at techservice@acciusa.com

Special incentive for switching to alternative solution: Available upon request contact techservice@acciusa.com

bioMérieux

Main manufacturing site: BioMérieux SA, France

Supply Worldwide through direct subsidiaries or distributor, Available upon request at: www.biomerieux.com

Training materials, Technical eLearning modules, white paper, references: bioMérieux | Pharma QC Endotoxin Testing

Peer review studies, Available upon request at: bioMérieux | citation list

Charles River Laboratories

Main manufacturing site: Charleston, SC USA
Website & Technical Information available at: www.criver.com/BET
endosafesupport@crl.com

LONZA

Main manufacturing site: Lonza Bioscience Walkersville, MA, USA

Supply Worldwide through direct subsidiaries or distributor: Available upon request at: Lonza Bioscience | Lonza

Training materials: Technical eLearning modules, white paper, references: QC Insider® Toolbox (lonza.com)

Peer review studies: Available upon request at : reference list - QC Insider® Toolbox (lonza.com)

Special incentive for switching to alternative solution: free eLearning modules (reference to this letter)

ACC

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Guide for Implementation of Recombinant Cascade Reagent (rCR) PyroSmart NextGen® for Bacterial Endotoxins Testing (BET) When Testing Per USP in Biopharmaceutical Manufacturing

In an era where secure supply chain and sustainability are paramount, the implementation of PyroSmart NextGen® (PSNG) - recombinant Cascade Reagent (rCR) for endotoxin testing is a necessary step forward. Designed for QC managers, QC directors, Regulatory Affairs professionals, and Sustainability Officers, this guide provides a roadmap for adopting rCR in your quality control systems.

Disclaimer: The information provided in this document is given for the purposes of education and discussion. It is not intended to be, and it should not be used as, a substitute for regulation and regulatory guidance. Decisions and actions should be based on the relevant regulations, guidance documents and pharmacopeial chapters, not on this document. This guide provides information about implementation of PSNG reagent only which is the only widely published recombinant cascade reagent commercially available. The analytical performance of other recombinant cascade reagents should not be assumed to be identical to PSNG.

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Why Implement PyroSmart NextGen® (PSNG) for BET Now?

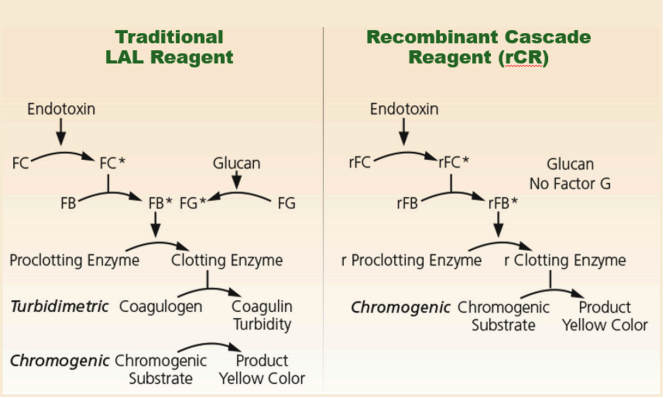
Implementing PSNG offers several compelling benefits:

- **Regulatory acceptance:** a long-awaited regulatory acceptance of recombinant cascade reagent with the *US Pharmacopeia* will go into effect for early implementation on 01 NOV 2024.
- **Non-animal derived reagents:** It reduces global dependency on wild marine arthropods as the raw material for a critical quality control test.
- **Sustainable and responsible production:** Allows aligning with global sustainability goals and the 3R ethical guidelines (Replacement, Reduction, Refinement).
- **Mitigation of Supply Chain Risk:** Globally, over 70 million BET tests are performed per annum, with 90% of all tests done using LAL reagents. Growing restrictions to harvest horseshoe crabs together with the growing demand for medicines create a supply chain risk.
- **Technical advancements:**
    1. Documented superior analytical performance: lot-to-lot reproducibility
    2. Proven specificity to endotoxins only, no cross-reactivity with 1,3-beta glucans
    3. Documented suitability for a wide range of products, including lower degree of interference in many products
    4. Documented specificity to all endotoxins, including those with four fatty acid chains within Lipid A structures
    5. A rapid assay with a shorter Time to Result than LAL assays
- **Seamless Integration:** Compatible with existing photometric platforms, allowing for straightforward incorporation into existing BET procedures.
- **Standard of Quality:** Produced under rigorous cGMP conditions in FDA-licensed facility for manufacture of Limulus Amebocyte Lysate (LAL).
- **Compatibility with automation of BET:** A proven reagent to adopt for high sample throughput productions.

A new general Chapter *<86> Bacterial Endotoxins Test Using Recombinant Reagents* will be published for early adoption on 01 NOV 2024 in the *US Pharmacopeia–National Formulary;* it will become official in May 2025. This chapter provides information on additional techniques to the current tests described in general Chapter *<85> Bacterial Endotoxins Test*. It includes methods using non-animal derived reagents, such as recombinant cascade reagent and provides information on how to incorporate them into their quality systems. Both USP *<86>* and *<85>* are compendial standalone chapters.

Principle of PyroSmart NextGen®

PSNG contains 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: a quantitative enzymatic assay based on the measure of change in absorbance at 405nm which is proportional to the presence of bacterial endotoxins. The kinetic chromogenic technique is included in USP Chapter *<85>* and Pharm Eu *2.6.14* Method D. Therefore, the stoichiometry and output of PSNG is well understood, published and accepted as a compendial technique. The dynamic range of PSNG is 50 to 0.001 EU/mL depending on the type of existing absorbance reader.



Key Differentiators Between LAL and PSNG

Understanding the differences between Limulus Amebocyte Lysate (LAL) and PSNG recombinant cascade reagent (rCR) is crucial when performing validation studies:

- **Source Material:** LAL is derived from horseshoe crabs, whereas PSNG is produced through recombinant DNA technology.
- **Specificity:** PSNG provides specificity to endotoxins only, no cross-reactivity with 1,3-beta glucans due to the absence of Factor G.
- **Composition:** PSNG formulation is based on decades of research and trials conducted by Seikagaku/ACC endotoxin experts. It reflects the most current knowledge of endotoxin biochemistry. Unlike LAL, PSNG formulation does not contain additional blood-derived proteins and peptides that upregulate the blood clotting mechanism in horseshoe crabs.

STEP 1: User Requirements

Successful implementation of PSNG rCR necessitates a clear understanding of user requirements in order to ensure an effective transition within quality control processes. This is achieved by producing a User Requirement Specification document. Meeting the user requirements will be demonstrated and/or tested as part of the validation protocol.

STEP 2: Equipment, Consumables and Resources Required

Successful implementation of PSNG rCR necessitates a clear understanding of specific user needs that ensure an effective transition within quality control processes:

- **Equipment**

Equipment	Available Yes/No
Incubating absorbance reader: microplate reader or Pyros Kinetix Flex® tube reader	
Absorbance detection at 405nm wavelength	
Current software built with settings needed for PSNG	
A set of pipettes (P20, P100, P1000)	
Repeater or multichannel pipette	
Vortex mixer	

- **Consumables**: All must be certified free of interfering endotoxins and must be meet the vendor's requirements

Consumables	Available Yes/No
PSNG lot matched with CSE lot (Certificate of analysis available)	
Reaction vessels (i.e. Pyroplates 96well plate for microplate readers or 8x75mm glass reaction tubes for tube readers)	
Dilution tubes	
Pipette tips	
Repeater Combit-tip (for repeaters) or a reservoir (for multichannel pipette)	
LAL Reagent Water	

- **Protocol Development:** Adopting SOPs for LAL assay for use of PSNG which should include procedure for initial qualification of the reagent and control standard endotoxin (CSE), implementation plan and implementation protocol. Should include required specifications.
- **Training and Education:** Training the analyst on the best practices of reagent handling and assay preparation, emphasizing the differences between the in-house LAL and PSNG reagent.
- **Ongoing Technical Support and Additional Resources:** Access to Global Technical Services (available in local time and languages) regarding any specific implementation concerns and troubleshooting:
  - ***US:*** *techservice@acciusa.com*

STEP 3: Risk Analysis

Before proceeding with implementation, conduct a sample-specific risk assessment relevant to bacterial endotoxin based on:

- Sample Type:
  - *Evaluation of the regulatory barrier for each sample based on their regulatory status (whether monographed or non-monographed)**
  - *Evaluation of the likelihood of endotoxin contamination (based on historical data sets and manufacturing process)***
  - *Evaluation of severity of endotoxin contamination***
    - *Process Microflora: corroborating data (i.e. Bioburden data and associated identification of the bacteria; Total Organic Carbon data for purified waters) may be used to determine the occurrence of the most sever risk: false negative results being reported by PSNG.*

Output of risk analysis is grouping samples into three categories:

- **No regulatory threshold samples**
- **Low regulatory threshold samples**
- **High regulatory threshold samples**

Implementation will be driven by output of the risk analysis.

Phase 1 Implementation – No Regulatory Threshold Samples

If already using one of the LAL kinetic photometric techniques (turbidimetric or chromogenic), the end user can seamlessly switch to PSNG with *no additional* testing requirements. The end user shall follow the general expectations as defined in USP <*85*> under Preparatory Testing and USP <*1085*> under Method Suitability Testing:

1. Assurance of the criteria for the standard curve when using PSNG and CSE

Minimal criteria	Setup	Required specifications
Standard series	3 concentrations in triplicate	$\|R\| \geq 0.980$
Negative controls	LRW in triplicate	Onset time > onset time of the lowest standard
Notes:	1 lot of each PSNG and CSE	

*Detailed information can be found in Appendix 1

**Detailed information can be found in Appendix 2

2. Method suitability testing when using PSNG: must be demonstrated for each product individually

Minimal criteria	Setup	Required specifications
Standard series	3 concentrations in duplicate	|R| ≥ 0.980
Negative controls	LRW in duplicate	Onset time > onset time of the lowest standard
Product	Series of dilutions not exceeding MVD in duplicate	< Endotoxin limit
Positive Product Controls (PPC)	Series of dilutions spiked with CSE to the middle of standard series in duplicate	PPC recovery % = 50 – 200%
Notes:	1 lot of each PSNG and CSE 1 lot of product	

3. Test for Interfering factors when using PSNG: must be demonstrated for each product individually

Minimal criteria	Setup	Required specifications
Standard series	3 concentrations in duplicate	|R| ≥ 0.980
Negative controls	LRW in duplicate	Onset time > onset time of the lowest standard
Product	A single dilution (< MVD) expected to yield a valid PPC in duplicate	< Endotoxin limit
PPC	A single dilution spiked to the middle of the standard series in duplicate	PPC recovery % = 50 – 200%
Notes:	1 lot of each PSNG and CSE 3 lots of product	

Note: If currently using one of the LAL photometric methods for testing the specific samples, the method suitability with PSNG is likely to be the same for that sample.

Note 2: If currently using the gel clot test only, prior to Preparatory testing, the end user must install and validate one of the platforms for kinetic chromogenic measure (i.e. Absorbance microplate reader powered by appropriate software).

Phase 2 Implementation – Low Regulatory Threshold Samples

On 01 NOV 2024, if already using one of the LAL kinetic photometric techniques (turbidimetric or chromogenic), the end user can seamlessly switch to PSNG with some additional testing requirements (early implementation). The magnitude of the additional testing should be based on consultation with local regulators.

Typically, the validation study includes:

- General expectations as defined in USP <*86*> under Preparatory Testing and USP <*1085*> under Method Suitability Testing (for details, see above):

1. Assurance of the criteria for the standard curve when using PSNG and CSE

Minimal criteria	Setup	Required specifications
Standard series	3 concentrations in duplicate	|R| ≥ 0.980
Negative controls	LRW in duplicate	Onset time > onset time of the lowest standard
Notes:	1 lot of each PSNG and CSE	

2. Method suitability testing: must be demonstrated for each product individually (see under Phase 1)

3. Test for Interfering factors: must be demonstrated for each product individually (see under Phase 1)

For non-monographed products - Steps 1., 2. and 3. complete the validation process.

For monographed products - additional requirements apply:

4. Obtain primary method validation package for PSNG from ACC (relevant publications under References and FDA Master File (in preparation))

5. Verification testing (suitability for the intended use) per USP <1226> Verification of Compendial Procedures: must be demonstrated for each product individually under actual conditions of use

6. Equivalency testing to the in-house LAL method: must be demonstrated for each product individually

Example

- **Verification of the accuracy and precision** of endotoxin detection in **WFI** and comparison of the results to the current compendial method to assess **equivalency** to LAL.
- Experimental design:

Criteria		Setup	Required specifications
Standard series	4 concentrations in triplicate (i.e. 10 – 0.01 EU/mL)		|R| ≥ 0.980
Negative controls	LRW in triplicate		Onset time > onset time of the lowest standard
WFI	Undiluted in duplicate spiked with 2 concentrations of CSE (high, low) (i.e. 5 and 0.05 EU/mL)		Accuracy: 50 – 200% Repeatability: CV% ≤ 30%* Intermediate precision: CV% ≤ 30%* *based on concentration
	1 assay includes multiple samples from different points of use		
Notes:	Different points of use 3 lots of each PSNG 1 lot of CSE 1 lot of LAL 2 analysts		
Example of a testing schedule	Day 1	Analyst 1	PSNG lot 1
			LAL lot 1
	Day 2		PSNG lot 2
			LAL lot 1
	Day 3		PSNG lot 3
			LAL lot 1
	Day 4	Analyst 2	PSNG lot 1
			LAL lot 1
	Day 5		PSNG lot 2
			LAL lot 1
	Day 6		PSNG lot 3
			LAL lot 1

Phase 3 Implementation – High Regulatory Threshold Samples

Even when USP <*86*> becomes official on 01 MAY 2025 in *USP* **2025 Issue 1**, USP <*86*> techniques will continue to be considered alternative procedures for monographed products (e.g. legacy products).

USP Perspective on Implementation of Alternative Methods or Procedures: The USP has long provided mechanisms for the implementation of alternative procedures to analyze compendial articles. Per USP General Notices and Requirements, **6.30. Alternative and Harmonized Methods and Procedures:** *"An alternative method or procedure is defined as any method or procedure other than the compendial method or procedure for the article in question. The alternative method or procedure* ***must be fully validated*** *(see USP <1225> Validation of Compendial Procedures* ***and must produce comparable results to the compendial method or procedure within allowable limits established on a case-by-case basis."***

Regarding legacy products, USP <*1223*> *Validation of Alternative Microbiological Methods*, mentions that ***"if a product has proven safe in widespread use when released or controlled using current methods, the implementation of an alternative method which can be well-correlated to the existing method should be straightforward."***

PSNG has been shown well-correlated to existing LAL assays (9, 10, 11, 12, 13). Therefore, if already validated by one of the LAL kinetic photometric techniques (turbidimetric or chromogenic), the end user can still seamlessly switch to PSNG with *some additional* testing requirements listed in USP <*86*> Draft as follows:

1. Assurance of the criteria for the standard curve when using PSNG (for details, see Phase 2 Implementation)
2. Method suitability testing when using PSNG: must be demonstrated for each product individually (for details, see Phase 2 Implementation)
3. Test for Interfering factors when using PSNG: must be demonstrated for each product individually (for details, see Phase 2 Implementation)
4. Obtain primary method validation package for PSNG from ACC (relevant publications under References and FDA Master File (in preparation))
5. Submit Prior Approval Supplement – Comparability Protocol to the appropriate review division of the US FDA
6. Verification testing (suitability for the intended use) per USP <*1226*> *Verification of Compendial Procedures:* must be demonstrated for each product individually under actual conditions of use

7. Equivalency testing to the in-house LAL method: must be demonstrated for each product individually
8. Regulatory submission: Submit the results (after confirming the process with the appropriate review division) – possibly as reduced reporting category Supplement – Changes being affected or Special Report)

Per USP *<1226> Verification of Compendial Procedures: "The verification process for compendial test procedures is the* ***assessment of whether the procedure can be used for its intended purpose, under the actual conditions of use for a specified drug substance and/or drug product matrix.*** *Verification consists of assessing selected analytical performance characteristics, such as those that are described in USP <1225>, to generate appropriate, relevant data rather than repeating the validation process. Users of compendial analytical procedures are not required to validate these procedures when first used in their laboratories, but documented evidence of suitability should be established under actual conditions of use."*

Verification should assess whether the compendial procedure is suitable for the product matrix and should include an assessment of the effect of the matrix on the recovery of endotoxin from the drug product.

Example

- **Verify the accuracy and precision** of endotoxin in **sodium citrate injection** and compare the results to the current compendial method to assess **equivalency** to LAL (Kelley *et al.*, 2023)
- Experimental design:

<table>
<tr><th>Criteria</th><th colspan="2"></th><th>Setup</th><th>Required specifications</th></tr>
<tr><td>Standard series</td><td colspan="2">4 concentrations in triplicate</td><td></td><td>|R| ≥ 0.980</td></tr>
<tr><td>Negative controls</td><td colspan="2">LRW in triplicate</td><td></td><td>Onset time > onset time of the lowest standard</td></tr>
<tr><td rowspan="2">Sodium citrate injection</td><td colspan="2">MVD/5 in triplicate spiked with 3 concentrations of USP RSE (high, mid, low) (i.e. 5, 0.5 and 0.05 EU/mL)</td><td></td><td>Accuracy: 50 – 200%
Repeatability: CV% ≤ 30%
Intermediate precision: CV% ≤ 30%
Range</td></tr>
<tr><td colspan="3">1 assay includes 3 lots of sodium citrate injection</td><td></td></tr>
<tr><td>Notes:</td><td colspan="4">3 lots of sodium citrate injection
3 lots of each PSNG
1 lot of CSE
3 lots of LAL (if possible)
2 analysts
Daily testing: 1 assay with PSNG + 1 assay with LAL</td></tr>
<tr><td rowspan="12">Example of a testing schedule</td><td rowspan="2">Day 1</td><td rowspan="6">Analyst 1</td><td>PSNG lot 1</td></tr>
<tr><td>LAL lot 1</td></tr>
<tr><td rowspan="2">Day 2</td><td>PSNG lot 2</td></tr>
<tr><td>LAL lot 2</td></tr>
<tr><td rowspan="2">Day 3</td><td>PSNG lot 3</td></tr>
<tr><td>LAL lot 3</td></tr>
<tr><td rowspan="2">Day 4</td><td rowspan="6">Analyst 2</td><td>PSNG lot 1</td></tr>
<tr><td>LAL lot 1</td></tr>
<tr><td rowspan="2">Day 5</td><td>PSNG lot 2</td></tr>
<tr><td>LAL lot 2</td></tr>
<tr><td rowspan="2">Day 6</td><td>PSNG lot 3</td></tr>
<tr><td>LAL lot 3</td></tr>
</table>

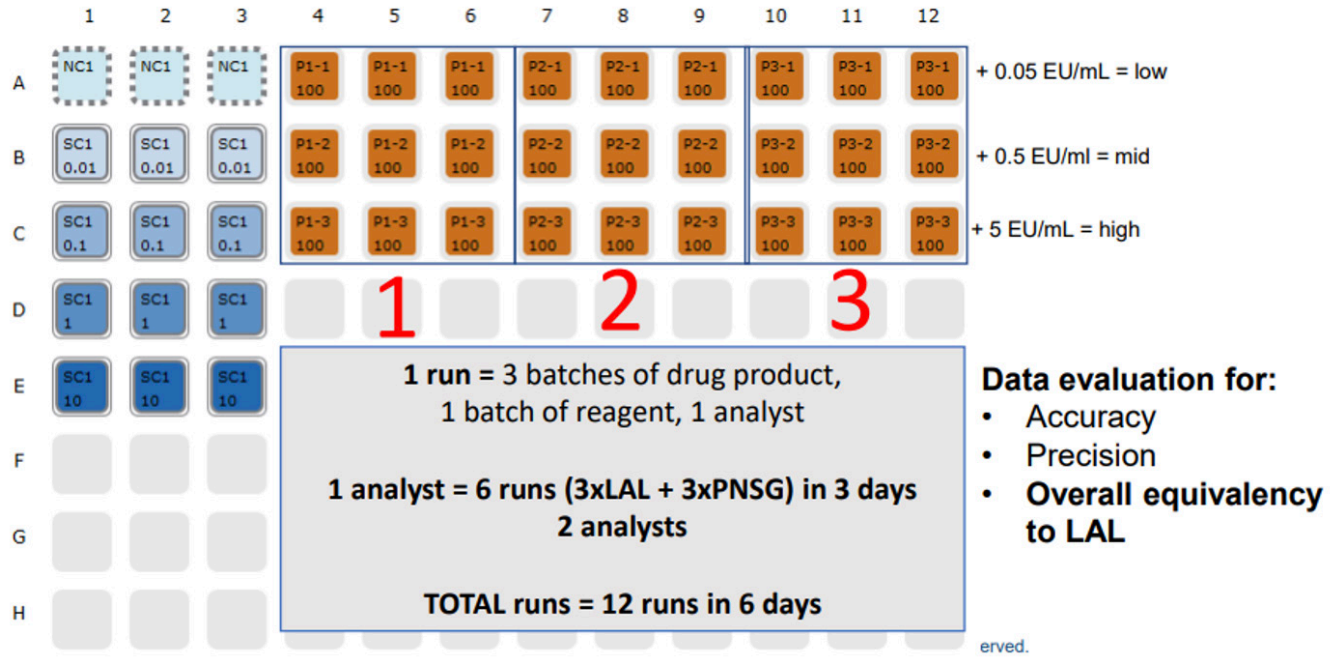


Figure 1 - An example of assay setup for verification and equivalency testing

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	$\|r\| \geq 0.980$
2. Accuracy (recovery)	**Standard Curve**	**Min-Max (%)**	**Min-Max (%)**	
	0.01 EU/mL	85-98	88-100	
	0.1 EU/mL	99-121	98-116	
	1.0 EU/mL	109-122	105-116	
	10 EU/mL	85-95	87-98	50-200%
	USP-RSE	**Min-Max (%)**	**Min-Max (%)**	
	0.05 EU/mL	109-118	95-115	
	0.5 EU/mL	116-131	107-127	
	5.0 EU/mL	116-134	130-153	
3. Precision				
3-1 Repeatability (CV)	**Standard Curve**	**Min-Max (%)**	**Min-Max (%)**	
	0.01 EU/mL	3-21	2-21	
	0.1 EU/mL	5-11	7-13	
	1.0 EU/mL	8-18	7-17	
	10 EU/mL	12-17	13-18	CV ≤30%
	USP-RSE	**Min-Max (%)**	**Min-Max (%)**	
	0.05 EU/mL	6-16	6-19	
	0.5 EU/mL	5-15	4-17	
	5.0 EU/mL	6-12	4-11	
3-2 Intermediate Precision (95% CI for CV)	**Standard Curve**	**Min-Max (%)**	**Min-Max (%)**	
	0.01 EU/mL	11-19	9-15	
	0.1 EU/mL	7-13	7-13	
	1.0 EU/mL	10-17	9-16	
	10 EU/mL	10-17	11-20	CV ≤30%
	USP-RSE	**Min-Max (%)**	**Min-Max (%)**	
	0.05 EU/mL	8-14	9-16	
	0.5 EU/mL	7-13	8-15	
	5.0 EU/mL	7-12	7-13	
4. Range		**0.01-10 EU/mL**	**0.01-10 EU/mL**	Precision, accuracy, and linearity at suitable level

Figure 2 - Published results of an example of assay setup for verification and equivalency testing

- Data analysis of analytical performance of PSNG in sodium citrate injection and equivalency comparison to LAL:
- Data summary:
  - *PSNG was verified to be suitable for BET testing (the intended purpose under actual conditions of use) in sodium citrate injection (specified drug matrix).*
  - *PSNG was statistically found to be equivalent or better than LAL in sodium citrate injection.*
  - *Sodium citrate injection is considered validated for release testing using PSNG.*

Conclusions

Implementing the recombinant cascade reagent for endotoxin testing is a strategic move towards greater sustainability, reliability, and efficiency in quality control processes. By following this guide, your organization can seamlessly transition to PSNG, maintaining high standards of accuracy and compliance while supporting environmental sustainability.

Ready to make the switch? Contact our team today to learn more about how PSNG can enhance your endotoxin testing processes.

Appendix 1 – Sample Types

Globally, over 70 million BET tests are performed per annum, with 90% of all tests done using LAL reagents. Out of the 70 million BET tests, about 75% of all tests are performed on water samples for pharmaceutical purposes. Therefore, transitioning all water testing alone from LAL to recombinant reagents can yield a dramatic reduction in the use of LAL.

Table 1: Estimation of typical samples tested in biopharmaceutical manufacturing

Samples	Percentage of sample types
Waters for pharmaceutical purposes	**75%**
Pharmaceutical ingredients	10%
Buffers and solutions	2%
In-process materials	3%
Finished products	10%

Water for Pharmaceutical Purposes

There are different water samples daily tested for endotoxin and used within biopharmaceutical manufacturing: monographed vs. non-monographed samples. Monographed samples have their specific monographs listed in the *US Pharmacopeia – National Formulary (USP-NF)* and some of them include the test for Bacterial Endotoxin under the list of specific tests required to be performed along with an endotoxin specification.

Note: Monographs contain tests, procedures, and acceptance criteria to ensure the identity, strength, quality, and purity of an article in addition to other requirements related to packaging, storage, and labeling. Monographs for parenteral products (injections and implanted drug products) also list under section Specific tests the requirement for BET along with the listed endotoxin limit.

All USP monographs, even after USP <*86*> becomes official on 01 MAY 2025, will continue to refer to the current reference only - USP <*85*>.

For example, heparin sodium injection is monographed where section for specific tests refers to USP <*85*> with an endotoxin specification of no more than (NMT) 0.03 EU/USP heparin units. Thus techniques described in USP <*86*> remain alternative techniques for BET assays in heparin.

A new parenteral product, newly developed and registered, does not have a dedicated USP monograph referencing a specific method but is still subject to bacterial endotoxin testing per USP <*1*> *Injections and implanted drug products (parenterals) – product quality tests: "All articles intended for parenteral administration should be prepared in a manner designed to limit bacterial endotoxins as defined in USP <85> or Pyrogen test <151>"*. Therefore, as of 01 NOV 2024, such products can be tested either by USP <*85*> or USP <*86*> methods.

Table 2: List of water samples per USP <1231> Water for Pharmaceutical Purposes - *monographed vs. non-monographed*

Samples	USP Monograph?	Reference to USP <*85*>?
Purified Water	Yes	No
Water for Injection (WFI)	Yes	Yes, < 0.25 EU/mL
Water for hemodialysis	Yes	Yes, < 1 EU/mL
Pure Steam	Yes	Yes, < 0.25 EU/mL
Sterile Purified Water	Yes	No
Sterile Water for Injection	Yes	Yes, < 0.25 EU/mL
Bacteriostatic Water for Injection	Yes	Yes, < 0.25 EU/mL
Sterile water for Irrigation	Yes	Yes, < 0.25 EU/mL
Sterile Water for Inhalation	Yes	Yes, < 0.5 EU/mL
Drinking water	No	N/A
Distilled water	No	N/A
Deionized water	No	N/A
Filtered water	No	N/A
High-purity water	No	N/A
Deaerated water	No	N/A

For example, sodium acetate injection does not have a USP monograph but as an injection is a subject to BET. The endotoxin limit is calculated using the limit formula per USP <*85*> and USP <*86*> as K/M (where K is the threshold pyrogenic dose and M is the maximum human dose). As of 01 NOV 2024, Both USP <*85*> and USP <*86*> will be considered compendial procedures for sodium citrate injection, if method suitability is demonstrated.

Table 3: Example of the projected status of USP <86> *based on monographed vs. non-monographed product types*

Samples	USP Monograph?	Reference to USP <*85*>?	Use of USP <*86*>
Pure steam	No	N/A	Use as compendial anytime
Purified Water	Yes	No	Use as compendial anytime
Sodium citrate injection	No	Yes, in USP <*1*>	Compendial as of 01 NOV 2024
Water for Injection (WFI)	Yes	Yes, < 0.25 EU/mL	Alternative even after 01 MAY 2025
Heparin sodium injection	Yes	Yes, NMT 0.03 EU/unis	Alternative even after 01 MAY 2025

Appendix 2 – Risk Assessment Examples

Table 4: Estimation of probability of endotoxin ingress per sample type based on validated manufacturing process and historical data sets

Samples	Probability of endotoxin ingress
Water for Injection (WFI)	**Very low**
Pharmaceutical ingredients	Low
Buffers and solutions	Low
In-process materials	Moderate
Finished products	Low

When combining the information gathered from the monographs and the risk analysis, all samples can be divided into three basic categories shown in Table 6.

Table 5: An example of Risk analysis of endotoxin ingress in pharmaceutical manufacturing per sample type

		Severity					
		Hazardous A	**High B**	**Moderate C**	**Low D**	**Minor E**	**Negligible F**
Probability	**Almost certain 7**	**HIGH RISK**					
	Very high 6						
	High 5						
	Moderate 4	4B – In-process materials			**LOW RISK**		
	Low 3	3A – Finished products		3 C – Ingredients			
	Very low 2			2C – WFI	2D – Buffers and solutions		
	Remote 1						

All water for pharmaceutical purposes samples fall into the No or Low regulatory threshold category, yet they are currently tested primarily by LAL (and typically using two of the most resource heavy methods (gel clot or turbidimetric assays).

Appendix 3 – Alternative Procedures

***USP* Perspective on Implementation of Alternative Methods or Procedures**

The *U.S. Pharmacopeia (USP)* has long provided mechanisms for the implementation of alternative assay methods or procedures to analyze compendial articles.

Per USP General Notices and Requirements

"6.30. Alternative and Harmonized Methods and Procedures

An alternative method or procedure is defined as any method or procedure other than the compendial method or procedure

Table 6: Final categories of sample types

Regulatory threshold	Sample types	Comments	Implementation timing and requirements
No	**Purified water** **Sterile purified water** Drinking water Distilled water Deionized water Filtered water High purity water Deaerated water	Non-monographed Low risk Annual reporting only	Implement anytime No additional test requirements, follow the principles of USP <85> for the kinetic chromogenic technique but using PSNG
Low	**Non-monographed** New parenteral finished products	High risk Early implementation: after 01 NOV 2024, USP <86> will be fully **compendial** Regulatory review and filing	Early implementation on 01 NOV 2024 No additional test requirements, follow the principles of USP <86> for the kinetic chromogenic technique using PSNG
	Monographed WFI, SWFI Pure steam Pharmaceutical ingredients Buffers and solutions	Even after 01 MAY 2025, USP <86> remains an **alternative** procedure Low risk Annual reporting only	Early implementation on 01 NOV 2024 Follow USP <86> plus three additional requirements: 1. Primary method validation package (vendor) 2. Verification (suitability for intended use) – product specific* 3. Equivalency testing – product specific* *The extent of 2. and 3. depends on local regulatory expectations
High	In-process materials Finished products	Monographed products: even after 01 MAY 2025, USP <86> remains an **alternative** procedure High risk Post-verification review and regulatory approval for legacy products	Early implementation on 01 NOV 2024 Follow USP <86> plus three additional requirements: 1. Primary method validation package (vendor) 2. Verification (suitability for intended use) – product specific 3. Equivalency testing – product specific

for the article in question. The alternative method or procedure ***must be fully validated*** (see *Validation of Compendial Procedures <1225>*) ***and must produce comparable results to the compendial method or procedure within allowable limits established on a case-by-case basis.*** Alternative methods or procedures can be developed for any one of a number of reasons not limited to simplification of sample preparation, enhanced precision and accuracy, improved (shortened) run time, or being better suited to automation than the compendial method or procedure. Only those results obtained by the methods and procedures given in the compendia are conclusive."

Per USP <*1223*> *Validation of Microbiological Methods:* "This statement allows considerable user latitude in the decision to use an alternative procedure for routine product release, provided that proper technical and scientific attention is paid to the selection, qualification, and implementation of the method. **If a product has proven safe in widespread use when released or controlled using current methods, the implementation of an alternative method which can be well-correlated to the existing method should be straightforward."**

Appendix 4 – References

1. *USP <85> Bacterial Endotoxins Test*
2. *USP <86> Bacterial Endotoxins Test using Recombinant Reagents - Draft - https://www.uspnf.com/sites/default/files/usp_pdf/EN/USPNF/usp-nf-notices/86-bacterial-endotoxins-tests-using-recombinant-reagents.pdf*
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4. *USP <1231> Water for Pharmaceutical Purposes*
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15. *PyroSmart NextGen® Package Insert*

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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.

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 2024 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.

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European Pharmacopoeia Approach to Testing for Pyrogenicity

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Introduction

The texts of the European Pharmacopoeia (Ph. Eur.) play a major role in ensuring the quality of medicines in Europe. They consist in general chapters and monographs, which are mandatory quality standards ubiquitously applied by the licencing authorities of the 39 signatory countries of the European Pharmacopoeia Convention and the European Union, with the overall aim of protecting public health. The European Pharmacopoeia Commission, the decision-making body of the Ph. Eur., is responsible for the elaboration and maintenance of its content. The European Directorate for the Quality of Medicines & HealthCare (EDQM) is a directorate of the Council of Europe and is entrusted with publishing the Ph. Eur. and bringing these standards to its users.

It goes without saying that any official standards dealing with the quality of medicines must address the issue of potential contaminants in the products concerned. Medicinal products contaminated with pyrogenic substances and administered parenterally may cause adverse reactions ranging from fever to life-threatening shock-like symptoms. The aim of pyrogenicity testing is to limit, to acceptable levels, the risk of these adverse reactions happening.

In the Ph. Eur., medicinal products are tested for pyrogenic substances according to general chapter *2.6.8. Pyrogens*. The test consists of measuring the rise in body temperature induced in rabbits by the intravenous injection of a sterile solution of the substance to be examined. The chapter was first published in the Ph. Eur. in 1971 and is still prescribed in a large number of monographs and general chapters.

Endotoxins from gram-negative bacteria (lipopolysaccharides) are the most common cause of pyrogenic reactions induced by contaminated pharmaceutical products. The level of bacterial endotoxins is verified using the procedures described in Ph. Eur. general chapters *2.6.14. Bacterial endotoxins* or *2.6.32. Test for bacterial endotoxins using recombinant factor C*, published for the first time, respectively in 1987 and 2020. These are the analytical methods most commonly used to address the pyrogenicity of medicinal products administered parenterally. They present the great advantage of avoiding the use of laboratory animals but the drawback of not detecting fever-inducing substances other than bacterial endotoxins.

There are, indeed, a small number of pyrogens that possess a different structure and that cannot be detected using the test for bacterial endotoxins. Such pyrogenic substances are detected using the procedures described in the general chapter Monocyte-*activation test (2.6.30*). The monocyte-activation test is therefore an *in vitro* pyrogen test that has the advantage not only of avoiding the use of laboratory animals, but also of being able to detect any pyrogenic substance, i.e. both endotoxin and non-endotoxin pyrogens.

Replacement of the Rabbit Pyrogen Test

The Council of Europe's *European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes* was opened for signature in 1986. Since that time, the Ph. Eur. Commission and its experts have carried out a program of work committed to Replacing, Reducing and Refining (3Rs) the use of animals for test purposes. Achievements have been significant,[1] but there are still challenges ahead. The Convention is referred to in a number of Ph. Eur. texts, including chapter *2.6.8: "In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. Wherever possible and after product-specific validation, the pyrogen test is replaced by the monocyte-activation test (2.6.30)."* In spite of this explicit instruction to replace the rabbit pyrogen test (RPT) by its *in vitro* alternative, the animal test continues to be widely used.

At its annual conference in 2018,[2] the European Partnership for Alternative Approaches to Animal Testing (EPAA) reported on a survey performed among European companies and testing institutes that still routinely perform the RPT and found that there is little incentive to perform alternative testing when a pyrogen test is prescribed in a monograph. The regulatory burden linked with the change to the in vitro test was also mentioned.

Reading the Ph. Eur. texts only, users reported a potential discrepancy between monographs and EU Directive 2010/63/EU:[3]

"Article 4

Principle of replacement, reduction and refinement

1. Member States shall ensure that, wherever possible, a scientifically satisfactory method or testing strategy, not entailing the use of live animals, shall be used instead of a procedure."

"Article 13

Choice of methods

1. Without prejudice to national legislation prohibiting certain types of methods, Member States shall ensure that a procedure is not carried out if another method or testing strategy for obtaining the result sought, not entailing the use of a live animal, is recognised under the legislation of the Union."

According to Article 13 of the directive, the instruction given in chapter 2.6.8 – to use an alternative to the animal test – should be applied systematically, but this is not done in practice.

In view of the situation, the complete removal of the RPT from the Ph. Eur. is necessary if the aim is to move towards the exclusive use of *in vitro* tests for the control of pyrogens.

Currently, chapter 2.6.8 is prescribed in 59 texts of the Ph. Eur.: three general monographs (including 2034 *Substances for pharmaceutical use*), three dosage form monographs (including *0520 Parenteral preparations*), three general chapters and 50 individual monographs, covering such diverse products as antibiotics, human vaccines and blood products. In June 2021, the Ph. Eur. Commission endorsed the strategy for the replacement of 2.6.8 in all of these 59 texts.[4] A new general chapter *5.1.13. Pyrogenicity* will be introduced in the Ph. Eur., which will provide guidance to help users decide on their own approach to pyrogenicity testing, based on a risk assessment: depending on the potential presence of non-endotoxin pyrogens, the user will have the choice between an *in vitro* pyrogen test or a test for bacterial endotoxins. Suppressed from all texts of the Ph. Eur., chapter 2.6.8 will no longer be an option and will ultimately be suppressed from the Ph. Eur. The whole exercise will take approximately 5 years and stakeholders will be consulted via the usual channels with, in 2023, the chance to consult all proposed revisions and the new general chapter *5.1.13* – currently under preparation – in Pharmeuropa online[5] and to comment as necessary.

Recombinant Factor C

The test for bacterial endotoxins uses, as its main reagent, the amoebocyte lysate from an animal, the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). Discussions among Ph. Eur. experts on the use of a synthetic alternative to this natural reagent, recombinant factor C (rFC), have been ongoing since 2006. It took over a decade to collect sufficient data for the method using the synthetic reagent to be described in the Ph. Eur. A major breakthrough came on July 1, 2020 with the publication of general chapter *2.6.32. Test for bacterial endotoxins using recombinant factor C* in the Ph. Eur.,[6] giving an official status to the procedure using the recombinant reagent. In January 2021, the procedure entered official use as a Ph. Eur. method. In April 2021, the EDQM broadcast a webinar on the bacterial endotoxin test using rFC, explaining its current status as an alternative to the bacterial endotoxin test using the amoebocyte lysate.[7]

General chapter *2.6.14. Bacterial endotoxins* gives a choice of six methods, A to F (gel-clot method: limit test, gel-clot method: quantitative test, turbidimetric kinetic method, chromogenic kinetic method, chromogenic end-point method, or turbidimetric end-point method), the Ph. Eur.'s aim would be to add a seventh method, method G, that could be used instead of any of the other methods. However, because the chapter has undergone International Harmonisation within the Pharmacopeial Discussion Group (PDG), no changes can be made to the chapter without the agreement of the other participating pharmacopoeias (United States Pharmacopeia

and the Japanese Pharmacopoeia).[8] The topic is currently under discussion within the PDG.

Animal Welfare

The question of animal welfare is often raised in the context of rFC. The Ph. Eur. approach to this issue is laid out in its Introduction: *"Use of animals. In accordance with the European Convention on the protection of animals used for experimental and other scientific purposes (1986), the Commission is committed to the reduction of animal usage wherever possible in pharmacopeial testing, and encourages those associated with its work to seek alternative procedures. An animal test is included in a monograph only if it has clearly been demonstrated that it is necessary to achieve satisfactory control for pharmacopeial purposes."* Strictly speaking, rFC does not fall within the scope of the above-mentioned Council of Europe Convention, as the horseshoe crab is not directly used in pharmacopoeia testing. Nonetheless and very importantly, rFC avoids the use of a reagent extracted from a natural source and endangered species. As a single molecular entity, it also has higher standardization potential and as such represents significant technological progress. Last but not least, there is the crucial question of supply of the reagent: with horseshoe crabs absent from its coastlines, for Europe, the use of a recombinant alternative avoids potential supply shortages and a dependency on non-European countries; the potential supply concerns prompted by complete reliance on a single natural resource (the horseshoe crab) must also be taken into account. The recombinant source is an obvious step towards independence in this regard.

The pyrogenicity project fits perfectly within the scope of the "Replacement" aspect of the 3Rs, i.e. "technologies or approaches which directly replace or avoid the use of animals in experiments where they would otherwise have been used." Although the replacement of animals is a significant achievement in itself, there will be additional benefits from changing from *in vivo* to *in vitro* tests, including increased scope for standardization and reduced variability which, together, constitute a significant technological advancement. The situation will be reviewed in five years, after the respective texts have undergone their revision process.

Conclusion

Over the last 50 years the Ph. Eur. has addressed the question of pyrogenicity using the analytical techniques available at the time, moving from animal tests towards *in vitro* methods and therefore promoting the use of standardized methods for a better control of medicines in Europe. The Ph. Eur. has recently engaged on a path that will put an end to the use of rabbits in pyrogen testing and increase the use of synthetic reagents for the detection of bacterial endotoxins.[4]

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The Truth of Endotoxin Values

Points for Consideration During Investigation of Aberrant BET Results

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Introduction

Bacterial Endotoxin Testing (BET) is mandatory for detection of Endotoxin/Lipopolysaccharides (LPS) in parenteral drug products. This is important as LPS is one of the most potent stimulants of the human innate immune system. Over the last decades the Limulus Amebocyte Lysate (LAL) test has been established as the gold standard for the detection of LPS. In order to qualify a test sample, the endotoxin activity is determined based on a standard curve using reference or control standard endotoxins. BET is described in international pharmacopoeias (e.g., Ph.Eur, JP, USP). Thus, the test does not need to be validated per se, however product specific verification with tests for interfering factors is required.

In routine, most samples are analyzed in duplicate using one verified dilution of the product. In order to exclude test interference (i.e., inhibition/enhancement of the enzymatic reaction) positive product controls (PPC) are performed in addition. When all system suitability tests fulfill provided acceptance criteria a result is considered to be valid. In case not all acceptance criteria are fulfilled or unexpected results are obtained, further investigations are needed. Sometimes, these aberrant BET results are hard to understand.

Within a quality-controlled BET laboratory, operator and laboratory errors are often investigated relatively fast. However, there are further more challenging aspects which can influence a test result. In order to better understand inconclusive BET results the following points are often discussed:

- Variation in LAL reagents
- Variation in standard curve
- Representativeness of standard endotoxins
- Activity of endotoxin
- Alteration of detectable endotoxin
- Effects of (1→3)-ß-D-glucans

Is a Difference in LAL Reagent Results Possible?

Yes. The LAL reagents are derived from horseshoe crabs and are therefore of biological origin. It has been described that the lysate is a relatively crude mixture

and is not a single purified enzyme. This means that the enzyme activity cannot be determined exactly for each lot of lysate manufactured. Furthermore, the manufacturing process includes the addition of buffers and detergents which contribute a further source of variability.[1] A reduced variability can be achieved by using recombinant Factor C reagents.

Can a Variation in the Standard Curve Effect the Test Result?

Yes. To quantify bacterial endotoxin, a standard curve is prepared in order to determine the endotoxin activity of a sample. Therefore, the quality of the standard curve is the basis of quantification. Using a linear standard curve, a change of only 1% in y-intercept can result in a change of up to 35% in measured endotoxin activity.[1]

In Figure 1, Y-intercept (Y-Achsenabschnitt) as a function of number of analysis (Analysennummer) from trending analysis is shown. All data points (full diamonds) fulfill the standard acceptance criteria. However, the typical observed variations may lead to increased/decreased test results depending on the y-intercept as small variations can lead to relative high variations in measured EU/mL.

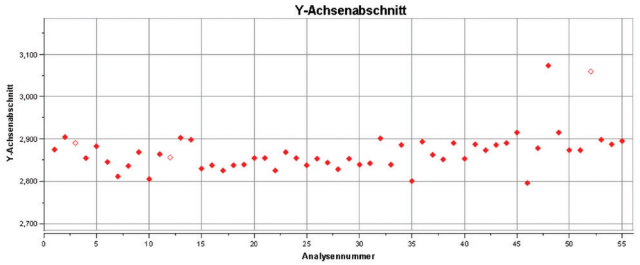

Figure 1 - Y-intercept of standard curves from trending analysis

Is Reference Standard Endotoxin Still Representative for BET?

Yes. Reference Standard Endotoxin (RSE) is the benchmark and allows comparability of test methods. Due to the heterogeneity of endotoxin, standardization of bacterial endotoxin tests was very challenging in the early time of BET. Only the introduction of RSE was the key factor to control the quality of BET, since *Limulus*-based approaches are ultimately biological assays, the lysates are intrinsically variable.[2] Moreover, recent challenges like LER and the implementation of recombinant tests brought up again discussions about Naturally Occurring Endotoxins (NOE). Advocates of NOE in the field of LER are refusing NOE when it comes to the comparison of test methods. It has been stated that NOE more closely mimics a real life contamination event,[3] but on the other hand it has been communicated that NOEs grown in laboratory are not representative of what occurs in nature. This contradictoriness clearly reflects the incongruous application of undefined endotoxin spikes during testing.

Table 1. Detection of endotoxin over time in a typical LER matrix

Low Endotoxin Recovery Study	[EU/mL]
M1 Time Point 0 days	64.0
M2 Time Point 1 days	27.8
M3 Time Point 2 days	17.6
M4 Time Point 3 days	7.3
M5 Time Point 7 days	4.8

The data is sourced from Low Endotoxin Recovery - Masking of Naturally Occurring Endotoxin[6]

Can a Sample Composition Alter the Detectability of Endotoxin?

Yes. There have been a lot of publications about Low Endotoxin Recovery (LER) and endotoxin masking which can lead to underestimation of endotoxin contents.[2,4–6] Due to the presence of certain excipients or active pharmaceutical ingredients or combinations thereof, endotoxin can be masked. An example for the detectability of endotoxin in a typical LER matrix is given in Table 1. Thereby the detectability decreases although the endotoxin is not degraded and potentially hazardous.

In order to reveal these effects so called LER studies are mandatory. Therefore, undiluted samples are spiked with endotoxin and held for a certain period of time. More guidance for LER including strategies for demasking is found in the Technical Report No. 82 from PDA.

Do (1→3)-ß-D-Glucans Affect the Endotoxin Test Result?

Yes. The LAL test includes per se the Factor G reaction pathway which is described to react with (1→3)-ß-D-glucans.[7] This reaction pathway has been identified years ago. Interestingly this pathway is unequally pronounced in different LAL tests. There are also agents available to repress Factor G reaction pathway. Unfortunately, it is neither proven that a) all glucans nor b) that their full activity is blocked. Obviously, glucans are very heterogeneous and present in various aggregation states and can be derived from a variety of sources. Once present in a sample the absolute differentiation between LPS and glucans with LAL is virtually impossible.

In the following example, a routine in-process control sample during drug manufacturing resulted in an unexpected endotoxin test result. With a routine chromogenic LAL test method, 6.5 EU/mL (Table 2, arithmetic mean) was determined. With a

turbidimetric LAL test method, 0.8 EU/mL (Table 3, arithmetic mean) was determined. The two test methods obtained valid results but with variations greater than the well-established 50% to 200%. Further analysis of (1→3)-ß-D-glucans revealed that the sample was contaminated by glucans (Table 4) which are most likely the root cause of the inconsistent results.

Table 2. Analysis of in-process control sample with chromogenic LAL test

Dilution	Measured value [EU/mL]	Endotoxin content [EU/mL]	PPC [%]	Status
1:5	1.47	7.35	143	Valid
1:10	0.675	6.75	118	Valid
1:20	0.271	5.42	175	Valid

Table 3. Analysis of in-process control sample with turbidimetric LAL test

Dilution	Measured value [EU/mL]	Endotoxin content [EU/mL]	PPC [%]	Status
1:5	0.141	0.705	176	Valid
1:10	0.098	0.980	147	Valid
1:20	0.0372	0.744	149	Valid

Table 4. Analysis of in-process control sample with (1→3)-ß-Dglucans test

Dilution	Measured value [EU/mL]	Endotoxin content [EU/mL]	PPC [%]	Status
1:50	59.799	2990.0	97	Valid
1:100	34.952	3495.2	74	Valid
1:500	5.922	2961.0	89	Valid

In order to determine the activity of endotoxin, only a recombinant reagent will allow determination of endotoxin, because of the lack of a Factor G reaction pathway.

Do Measured Activities Allow an Absolute Quantification of Endotoxin?

No. These test methods do not measure the amount of endotoxin/LPS, these tests rather measure activity (Endotoxin Units (EU)). The measurements quantify endotoxin activity which may vary from endotoxin to endotoxin. In the example below, supernatants of bacterial suspensions were analyzed using chromogenic LAL, recombinant reagent rFC and PBMC/IL6-based Monocyte Activation Test (MAT) (Table 5). While all tests were valid (i.e., according European Pharmacopoeia 2.6.14, 2.6.30, 2.6.32) and manufacturer instructions, LAL and rFC tests resulted in the same order of magnitude, MAT measured values approximately 100 times less in one sample. Although the test results substantially deviate in MAT, this result should not be judged as incorrect. MAT is based on the reactivity of human monocytes (e.g., Toll-like Receptor 4) and LAL/rFC is based on the reactivity of Horseshoe Crab Factor C.

Table 5. Analysis of supernatants of bacterial suspension using LAL, rFC and MAT

Sample	LAL [EU/mL]	rFC [EU/mL]	MAT [EU/mL]
Agrogbacterium radiobacter	207,000	242,500	2,000
Burkholderia multivorans	21,000	17,513	10,228

Considering this fact, the result from MAT seems to be more relevant regarding the proximity/relevance of the test method to a patient.

Conclusion

With respect to the examples provided it is difficult to rely on a single value. One single test method might not give the ultimate result. Although these tests methods have been used since decades, this does not imply that they can be used without considering their inherent advantages and disadvantages. Bacterial Endotoxin Tests are biological test systems and require careful interpretation as the relative detectability can vary more than the typical 50 % to 200 %.

Furthermore, application of the Monocyte Activation Test can be beneficial in providing more dedicated insights regarding the pyrogenic effects of a contamination. Generally, the test methods are only models to recapitulate the human situation. Despite all the challenges, available tests including recombinant tests are fast and sensitive methods to detect minute amounts of endotoxin.

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