

BACTERIAL ENDOTOXIN TESTING: PROGRESSIVE SCIENCE AND SUSTAINABILITY

JANUARY 2025



Evolution of Bacterial Endotoxins Testing

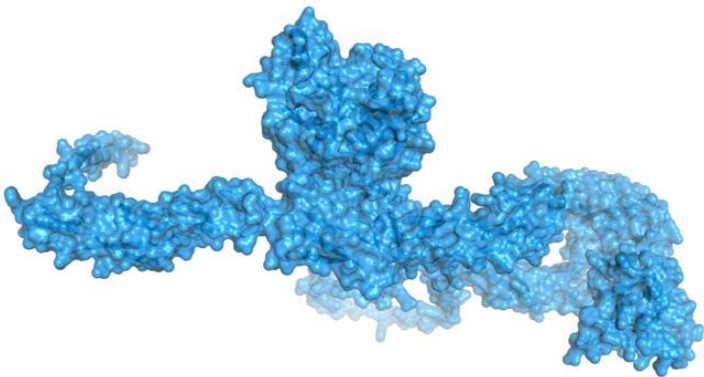


Rabbit Pyrogen Test



Bacterial Endotoxins Test: LAL & TAL

Image courtesy of Jack Levin



Recombinant Bacterial Endotoxins Test

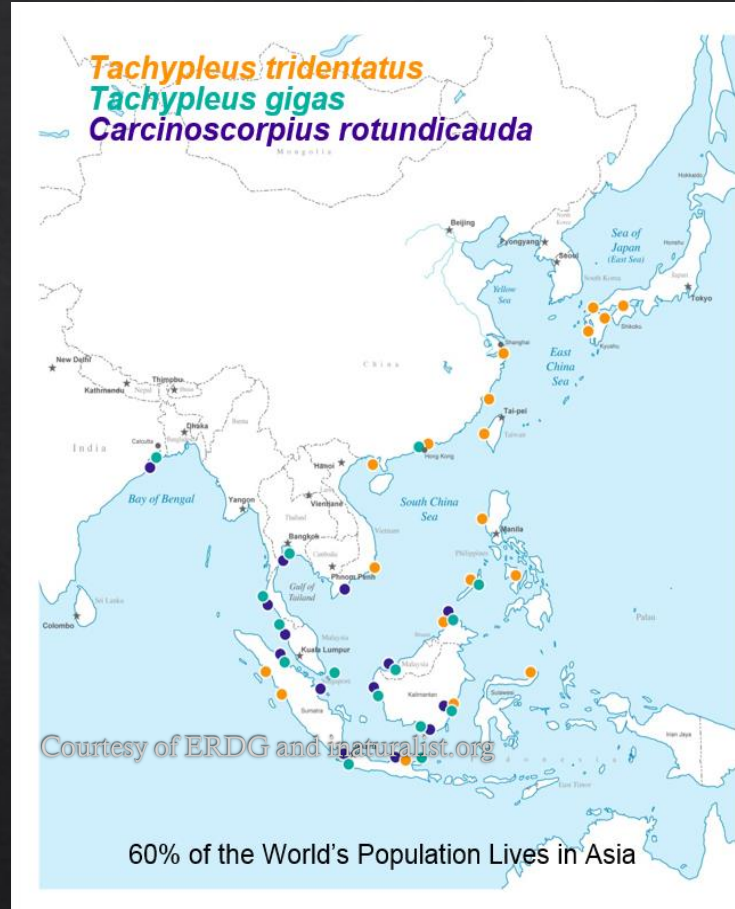
Image courtesy of Lynne Ding; Tim Cernak/Tesko Chaganti using AlphaFold3 from sequence in J. Genet. Eng. Biotechnol., 2023, 21, 44.

Horseshoe Crab Distribution

- ◇ *Carcinoscorpius rotundicauda*, the mangrove horseshoe crab
- ◇ *Limulus polyphemus*, the Atlantic or American horseshoe crab
- ◇ *Tachypleus gigas*, the Indo-Pacific, Indonesian, Indian or southern horseshoe crab
- ◇ *Tachypleus tridentatus*, the Chinese, Japanese or tri-spine horseshoe crab

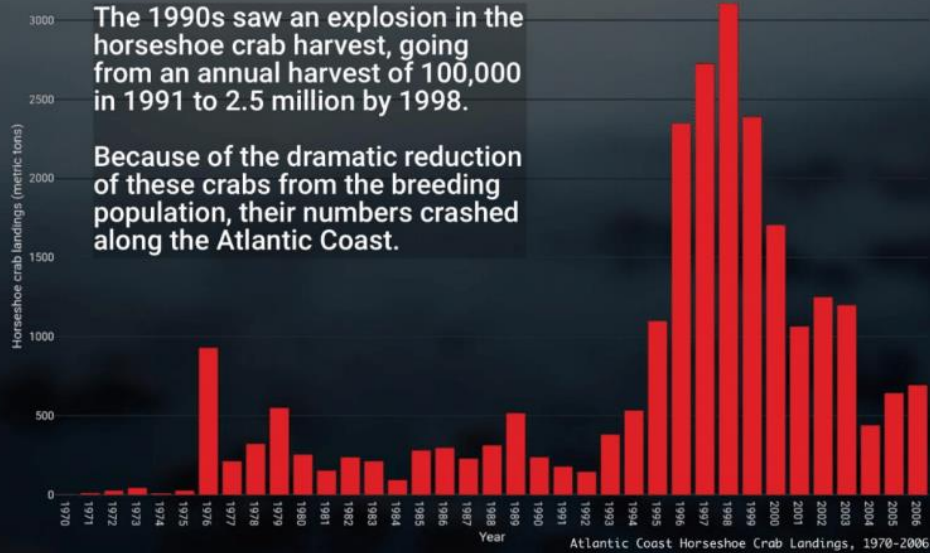


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The Limulus Crash

1) Overharvesting of horseshoe crabs led to a crash in their population.



In the early 1990s, horseshoe crabs became a popular bait for commercial fisheries, notably that of whelk and eel. The whelk fishery was booming as a domestic and international food, while eel was a popular bait choice for striped bass. Horseshoe crabs were found to be very effective bait for these species, and their demand skyrocketed. With fishers using 1-2 crabs per pot and a preference for gravid (egg-bearing) female crabs, the heavily increased harvest devastated horseshoe crab populations.

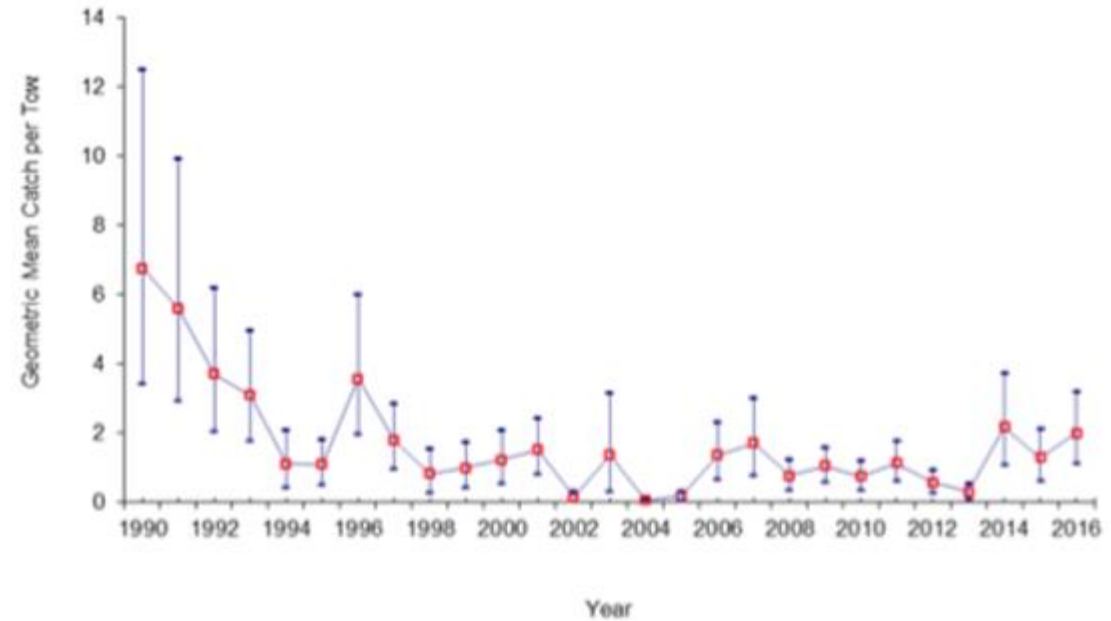


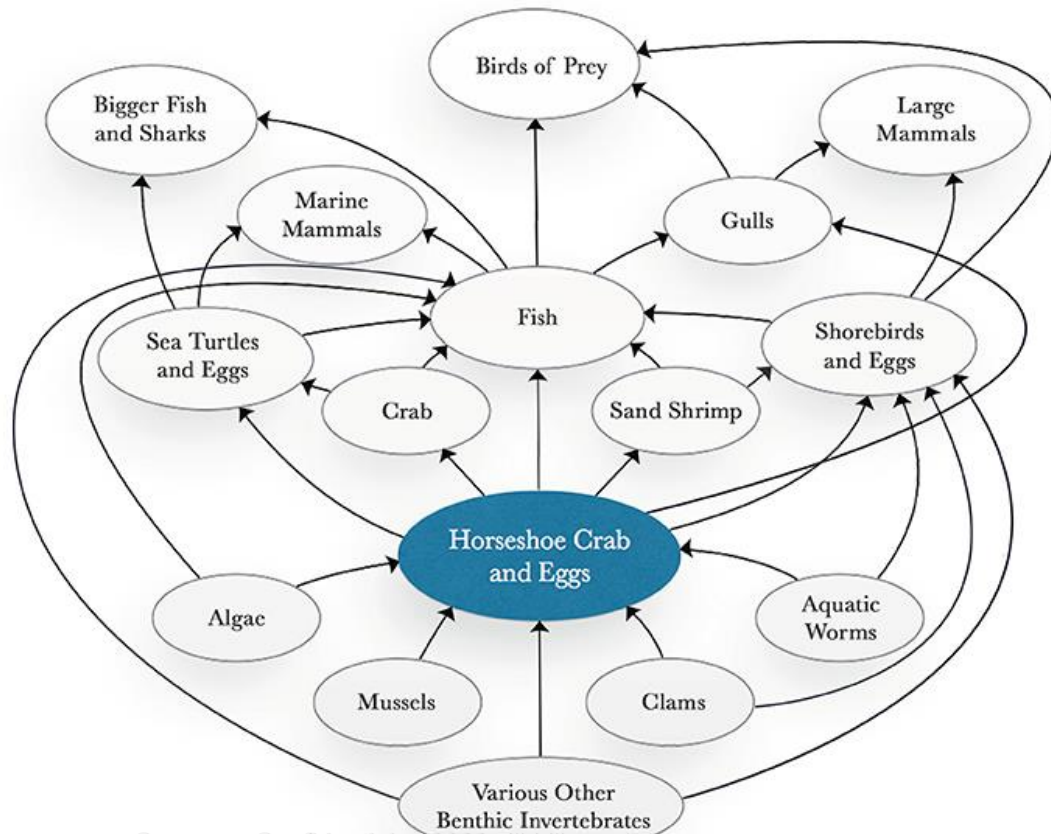
Figure e-1. Index of horseshoe crab relative abundance from Delaware's 30-foot trawl survey for the months April through July.

Delaware's ASMFC Horseshoe Crab Compliance Report for Fishing Year 2016, Report to ASMFC 2017

- ❖ The collapse of the Delaware Bay Horseshoe crab population in the late 1990's created an unprecedented corresponding collapse of shore bird populations which ultimately precipitated the Federal Listing of the Red Knot.
- ❖ By 2002 Horseshoe Crab numbers fell to historic lows

Shorebirds Depend on Horseshoe Crabs

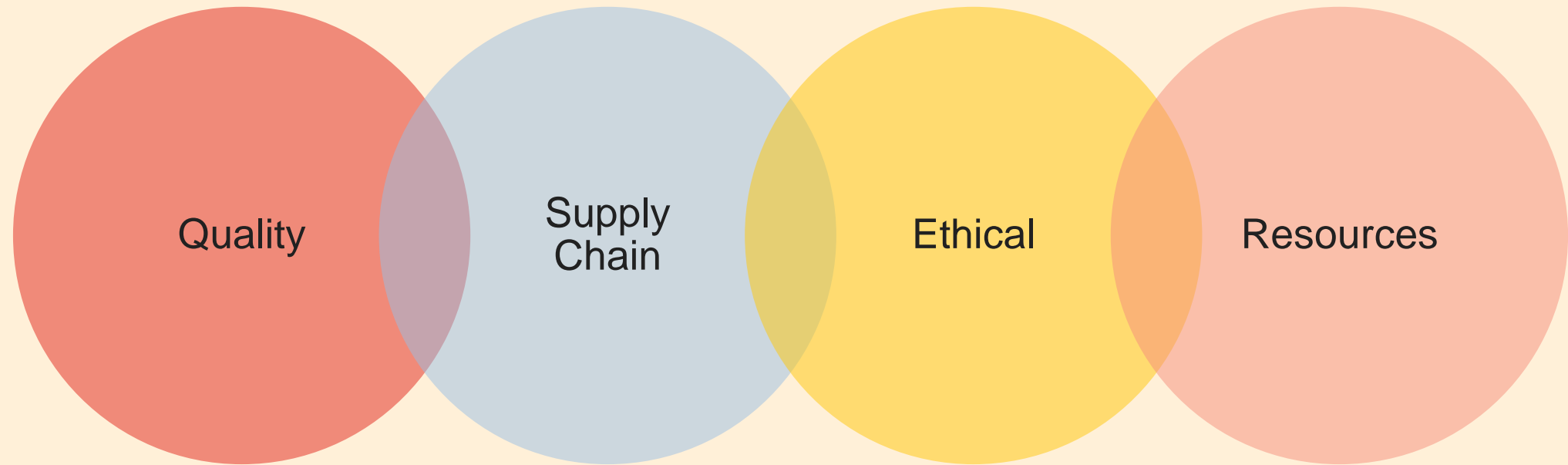
Horseshoe Crab Food Web



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From Krisfalusi-Gannon et. al.

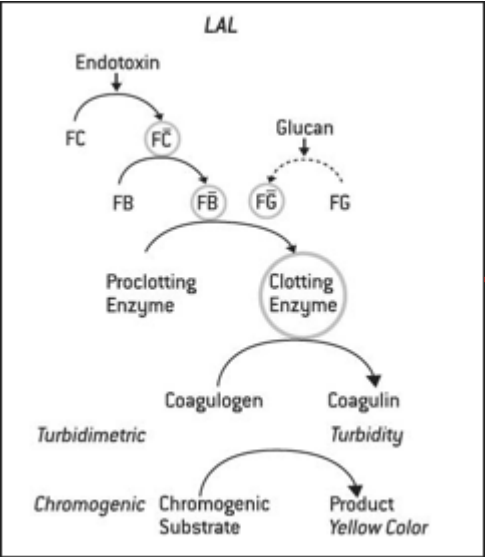


Change Rationale

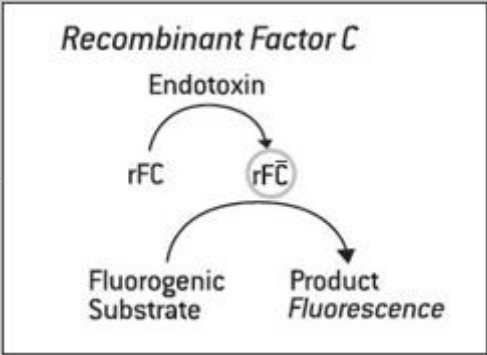


Recombinant Reagents are Biochemical Equivalents to LAL

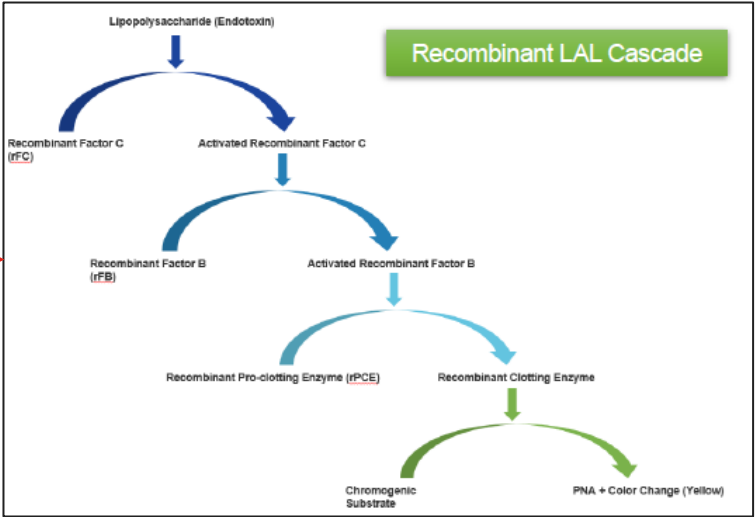
Mechanism of Action



Courtesy of Lonza PyroGene™ package insert



Courtesy of Lonza PyroGene™ package insert



Courtesy of Charles River / Endosafe® Trillium® package insert

Timeline



HISTORY

1997: Patented
2003: Commercially Available
2006: Lilly Evaluation

THE START

2012-2013:

- New Lilly China plant
- FDA Guideline
- USFWS lists Red Knot
- 2nd rFC supplier

THE LINE

2016:

- Generated evaluation data
- Validated method
- Line in the sand

SUCCESS

2018: First product approval

2020:

- Additional product approvals
- Ph.Eur. 2.6.32
- Tri-spine horseshoe crab declared endangered

FUTURE

2024:

- USP <86>
- Strive for full conversion
- Automation



rFC Equivalency: 2020 PDA Literature Review

- 14 peer reviewed studies from 2010 to 2018
- 1087 sample containing real world endotoxin
- 213 different relevant pharmaceutical products in 7 categories:
 - Large molecule/Peptide
 - Small molecule
 - Vaccine
 - Excipient / raw material
 - Container component
 - Device
 - Buffer / Water
 - Clinical
 - Plant Extract

- ❖ LAL and rFC are comparable for endotoxin detection
- ❖ rFC is superior to LAL for endotoxin specificity

Jay Bolden, Chris Knutsen, Jack Levin, Catherine Milne, Tina Morris, Ned Mozier, Ingo Spreitzer, Friedrich von Wintzingerode. Currently available recombinant alternatives to horseshoe crab blood lysates: Are they comparable for the detection of environmental bacterial endotoxins? A review. 2020. PDA Journal of Pharmaceutical Science and Technology, Volume 74, Issue 5, Pages 602-611

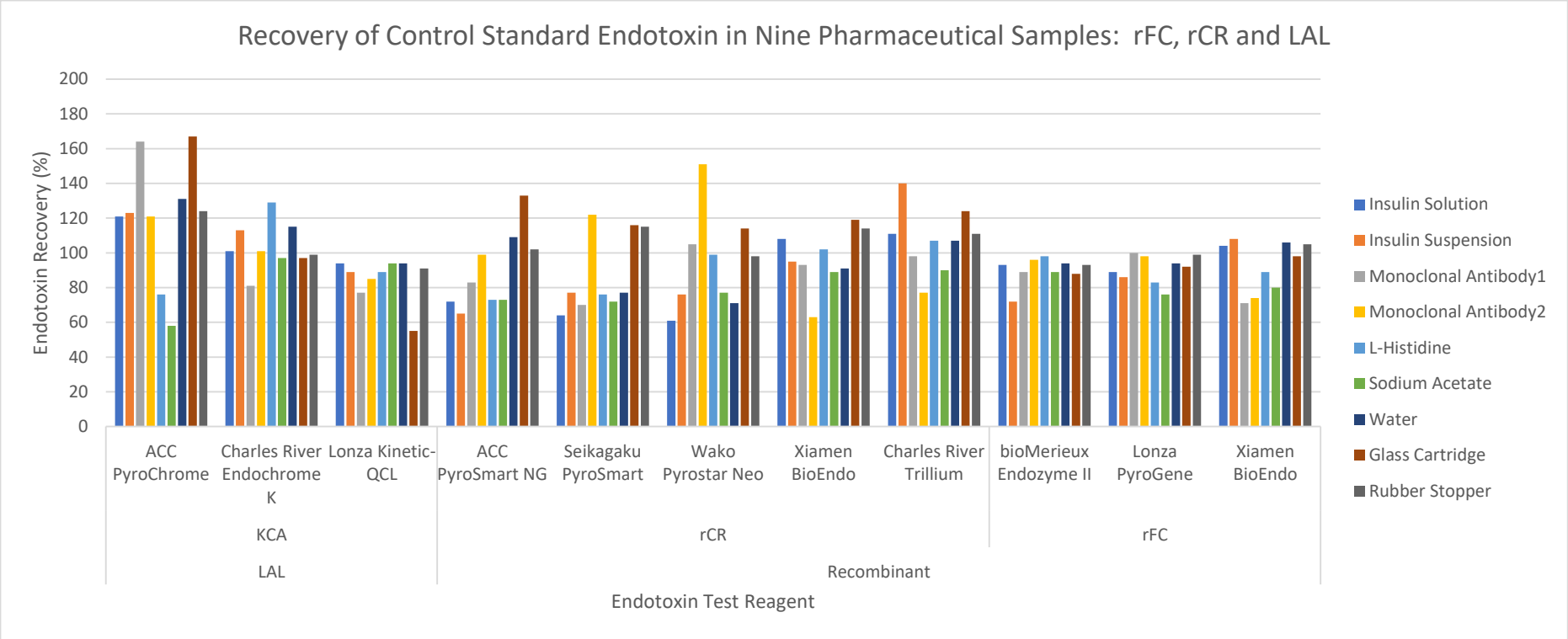
Environmental Endotoxin	Type	Source
3	Live Bacteria/Pharma	Bolden 2017
13	Pharma	Mozier
6	Environment	Kikuchi 2017
20	Environment	McKenzie
5	Pharma	Schwarz
912	Environment	Thorne
60	Environment	Alwis
1	Pharma	Marius
2	Pharma	Bolden USP
65	Clinical	Strachan
1087		

Recombinant Comparability

Recombinant reagents are equivalent or superior to LAL using 10,000 EU/vial national Reference Standard candidate lot endotoxin

Reagent	Average (EU/vial)	%CV
CR Gel Clot	1.58E+04	25%
CR KTA	1.20E+04	24%
Lonza LAL	1.15E+04	22%
Lonza rFC	1.05E+04	18%
BMX rFC	1.10E+04	19%
Xiamen rFC	9.90E+03	9%
Wako rCR	1.06E+04	28%
Xiamen rCR	9.99E+03	3%
Reagent	Average (EU/vial)	%CV
LAL	1.23E+04	27%
rFC	1.05E+04	17%
rCR	1.03E+04	21%
Recombinant (rFC + rCR)	1.04E+04	19%

Recombinant Comparability



Average of %PPC	
LAL	105
KCA	105
ACC PyroChrome	121
Charles River Endochrome K	106
Lonza Kinetic-QCL	90
Recombinant	93
rCR	94
ACC PyroSmart NG	91
Seikagaku PyroSmart	88
Wako Pyrostar Neo	91
Xiamen BioEndo	95
Charles River Trillium	107
rFC	90
bioMerieux Endozone II	89
Lonza PyroGene	89
Xiamen BioEndo	93
Grand Total	96

- Compendial recovery criteria is 50-200%; product dilutions were based on equivalent LOQs, not necessarily optimized for PPC recovery
- Theoretical recovery target is 90.9% based on a 10 µL “hot spike” into 100 µL sample



Compendial Status - Global

Compendial Method

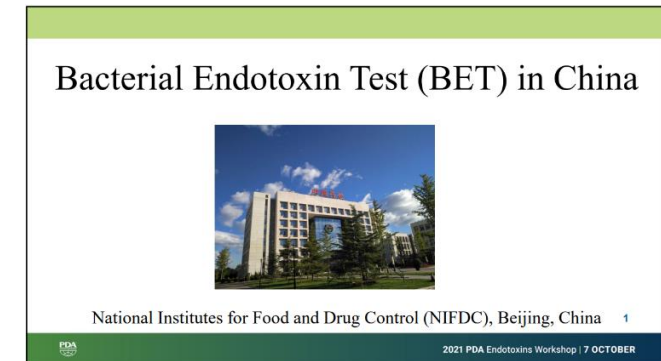
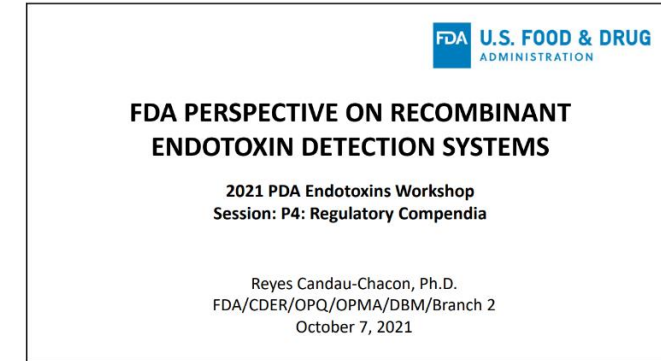
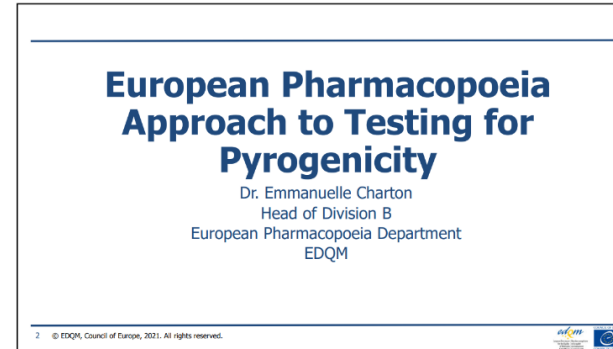
2020: Ph.Eur. 2.6.32
2022: EAEU (proposed)
2024: BP XIV C
2024: USP <86>

Compendial Monograph

2023: Ph.Eur. WFI

Compendia Guidance Chapter

2020: ChP 9251
2021: JP G4-4-180
2023: KP



Add the following:

▲(86) BACTERIAL ENDOTOXINS TEST USING RECOMBINANT REAGENTS

USP <86>

The Bacterial Endotoxins Test (BET) described in this chapter contains additional techniques using nonanimal derived reagents to the [Bacterial Endotoxins Test \(85\)](#). Unless specified in an individual monograph, the tests in this chapter are considered alternative tests and must meet the requirements in [General Notices 6.30](#). This test uses a reagent containing the recombinant Factor C (rFC) protein or a recombinant cascade reagent (rCR) containing recombinant Factor C, recombinant Factor B, and recombinant proclotting enzyme. These reagents are used to detect or quantify endotoxins from Gram-negative bacteria in test samples. The test is performed using reagent(s) based on the gene sequence(s) of the relevant factors of the horseshoe crab (*Limulus polyphemus*, *Tachypleus tridentatus*, or *Carcinoscorpius rotundicauda*).

There are two detection techniques that can be employed in this test: the endpoint fluorescence technique, based on the development of fluorescence after activation of a synthetic peptide-fluorophore complex; or the chromogenic technique, based on the development of color after cleavage of a synthetic peptide-chromophore complex. To accurately detect endotoxins, the test is carried out using endotoxin-free materials with laboratory controls in place to prevent inadvertent endotoxin contamination.

It is the responsibility of the user to review the supplier's primary validation package and to verify that the recombinant reagent-based test is appropriate for use in the intended application. The user should confirm that the reagent is suitable for its intended use by performing suitable experiments to confirm that the reagent meets the conditions of use for the material, and/or drug, and/or device, and/or combination thereof, being applied. The user should refer to [Verification of Compendial Procedures \(1225\)](#). Regulatory authorities may require supplemental data prior to acceptance, and users are encouraged to consult each regulatory authority. An example of supplemental data may include a comparative study of the material tested by techniques described in this chapter and those in [\(85\)](#).

APPARATUS

Depyrogenate all glassware and other heat-stable materials in a hot air oven using a validated process. A commonly used minimum time and temperature is 30 min at 250°. If employing plastic labware, such as microplates and pipet tips, use labware that is shown to be free of detectable endotoxin and does not interfere with the test.

REAGENTS AND TEST SOLUTIONS

Reagents

Recombinant reagents are based on the gene sequences of the relevant factors of the horseshoe crab (*L. polyphemus*, *T. tridentatus*, or *C. rotundicauda*). All reagents, including the fluorogenic substrate and assay buffer, must be free of detectable endotoxin.

2019

- Proposed revision to <85> to include recombinant reagents

2020

- Proposed revision withdrawn based on stakeholder feedback
- Proposed guidance chapter <1085.1>

2021

- Published <1085.1> stakeholder feedback
- Proposed USP comparability study

2023

- New USP Microbiology Expert Committee
- Proposed <86> rBET

2024

- Announced approval of <86> and early adoption date for **01-Nov-2024**
- Proposed revision to <1085>

Challenges

Widespread use of Gel Clot LAL in
developing countries

Overcoming Technology Barriers

Potential Supply Chain Impacts

Compendial Harmonization

Regulatory Pressure



Anti-smuggling operation in October 2019, the Riau Police successfully intercepted a smuggling attempt of 1,500 horseshoe crabs

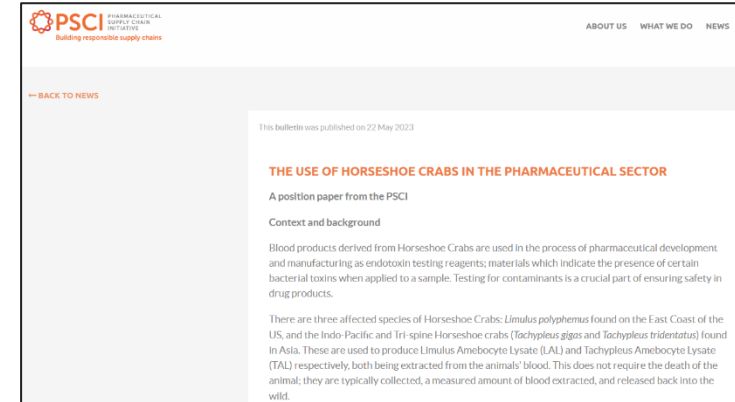
Horseshoe crabs from Palembang, South Sumatra to Medan, North Sumatra in February 2020.

460 *Tachypleus gigas* were confiscated from the local fisherman in Medan by special crime branch department in March 2020

"Global Trade Data and Conservation Challenges for Asian Horseshoe Crabs"
Akbar John, IUCN Horseshoe Crab SSC 2024

Opportunities

Cease use of TAL
Reduce LAL use
Implement rBET



Compendial Methods → de facto Harmonization
Regulatory Innovation and Consistency

Reducing
Horseshoe Crab
Crude Intensity

Gel-Clot
LAL



0%
reduction

Kinetic
Turbidimetric LAL

Zero reduction in horseshoe crab
blood from Gel-Clot

54%
reduction

Kinetic
Chromogenic LAL

54% reduction in horseshoe
crab blood from Gel-Clot

95%
reduction

LAL Cartridge
Technology

95% reduction in horseshoe
crab blood from Gel-Clot



100%
reduction

Recombinant Cascade
Reagent (rCR)

100% horseshoe crab blood free

Courtesy of Charles River

What is in your control
to change?

