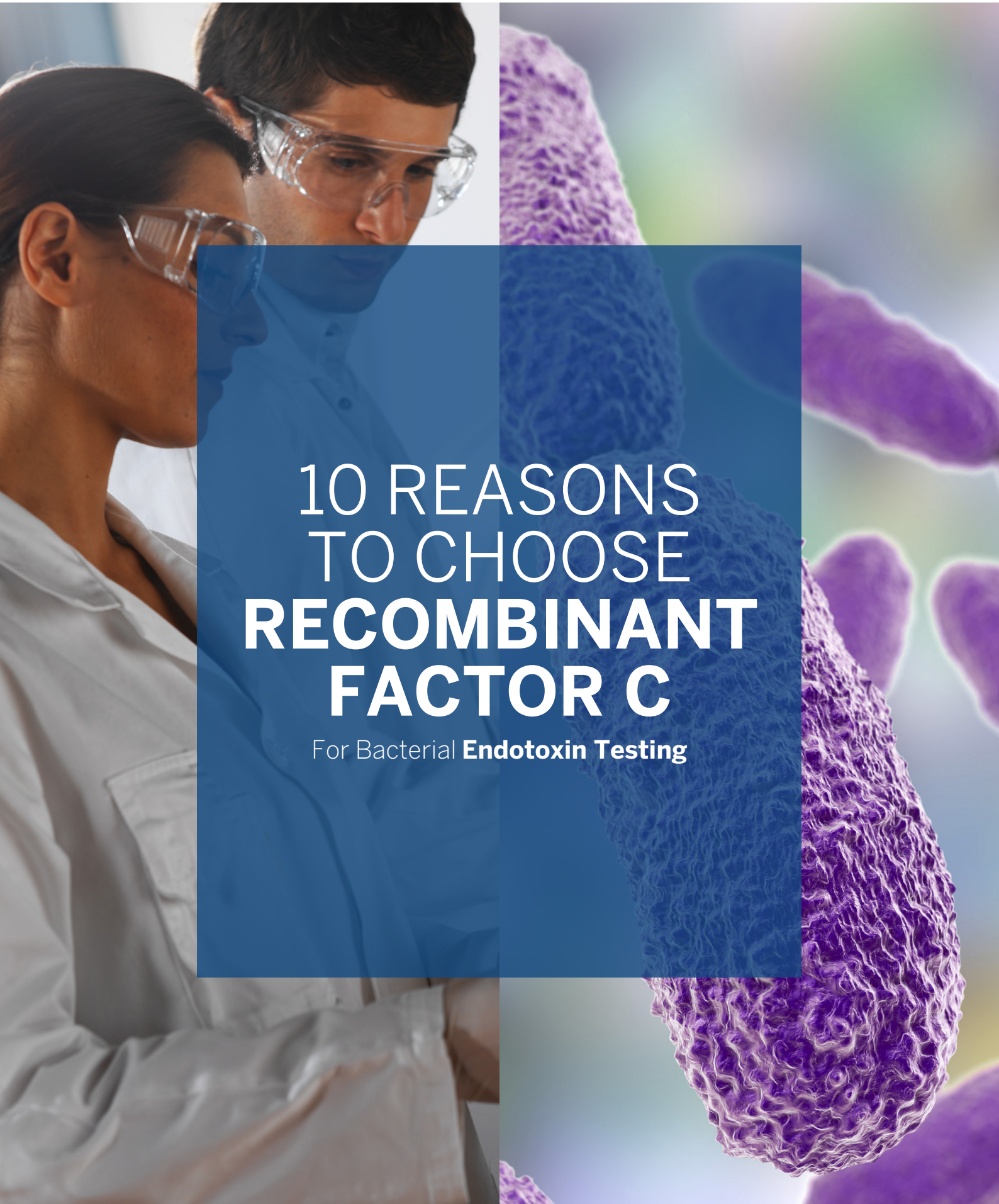
BIOMÉRIEUX



10 REASONS TO CHOOSE **RECOMBINANT FACTOR C**

For Bacterial **Endotoxin Testing**

Recombinant horseshoe crab Factor C (rFC) methods are the latest state-of-the-art solution for effective bacterial endotoxin testing (BET). This whitepaper reviews the advantages of Recombinant horseshoe crab Factor C (rFC) over the BET methods currently in widespread use. We compare the performance of LAL reagents with rFC, and summarize the evidence supporting our 10 reasons to choose rFC.

BACKGROUND

Macrophages, a type of immune cell, use the lipopolysaccharides (LPS) molecules that make up the outer cell wall of gram-negative bacteria to detect their presence in bodies. LPS, also known as bacterial endotoxins, can elicit a severe response in the immune system, resulting in fever, hypotension, nausea, shock, and sepsis.

Severe reactions to bacterial endotoxins can be fatal, and as such, great care must be taken to ensure that they don't find their way into medical products making contact with a patient's bloodstream or cerebral fluid.[1,2]

Unfortunately, avoiding endotoxin contamination during pharmaceutical manufacturing processes is by no means an easy task, because LPS molecules are present in virtually every environment. As a result, there are **strict regulations for the acceptable levels of endotoxin contamination** in medical devices, injectable pharmaceuticals, and other medical solutions that may come into contact with a patient's blood or cerebral fluid. Such products must, therefore, be tested for endotoxin contamination before they can be released.[3]

A BRIEF HISTORY OF ENDOTOXIN TESTING WITH LAL

In the 1960s, scientists discovered that the isolated lysate from the Atlantic horseshoe crab (**Limulus Amebocyte Lysate -LAL**) coagulated when in the presence of bacterial endotoxins. Around 10 years later, researchers also found that the same process occured in the isolated lysate from the Asian horseshoe crab (Tachypleus Amebocyte Lysate - TAL). Bouyed by their discovery, they devised tests aimed at detecting bacterial endotoxins using LAL and TAL reagents, which were then adopted by the US Pharmacopeia in 1983.[2,4]

Three approved main test methodologies utilize LAL and TAL reagents for endotoxin testing: Gel Clot (limit and semi-quantitative), Turbidimetric, and Chromogenic (end-point and kinetic methods) assays. The LAL/TAL tests all work in a similar fashion; the presence of bacterial endotoxins in a sample sets off a cascade of reactions, resulting in a change of turbidity or color (see Figure 1).[4,5]

The amebocyte lysate contains a mixture of naturally occurring proteins that are involved in the endotoxin detection process. Factor C acts as the principal biosensor by binding with bacterial endotoxins, activating another protein called Factor B. Factor B then converts a pro-clotting enzyme to a clotting enzyme. The resulting clotting enzyme then catalyzes a reaction that causes a change in viscosity, turbidity, or color, which is detected to determine the concentration of endotoxins in the sample.[4,5]

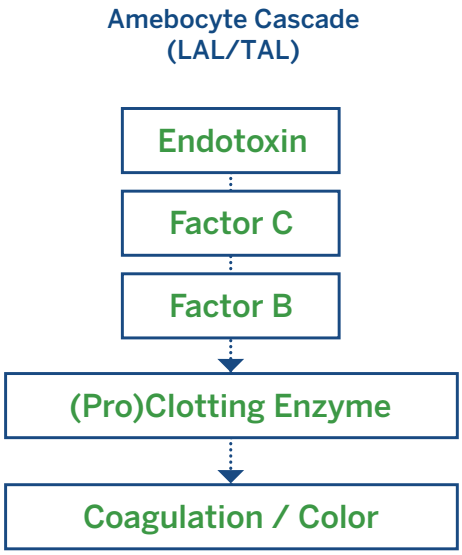


Figure 1: The presence of bacterial endotoxins sets off a cascade reaction in amebocyte lysates.

LAL: ENVIRONMENTAL IMPACTS AND LIMITATIONS

Although LAL and TAL are widely used in the pharmaceutical industry (principally because they provide sensitive detection of endotoxins), they have several disadvantages. LAL and TAL tests are susceptible to false-positives, and furthermore, the relatively high batch-to-batch variation found in natural lysate reagents reduces their reliability and comparability.[4,6]

Perhaps the most significant problem with LAL and TAL reagents is that they must be obtained from horseshoe crabs. The Atlantic horseshoe crab population has declined by 90% over the last 15 years, and the species is now described as vulnerable by the IUCN Redlist of threatened species. The Asian horseshoe crab population has been similarly affected, and is now considered endangered.[6,7]

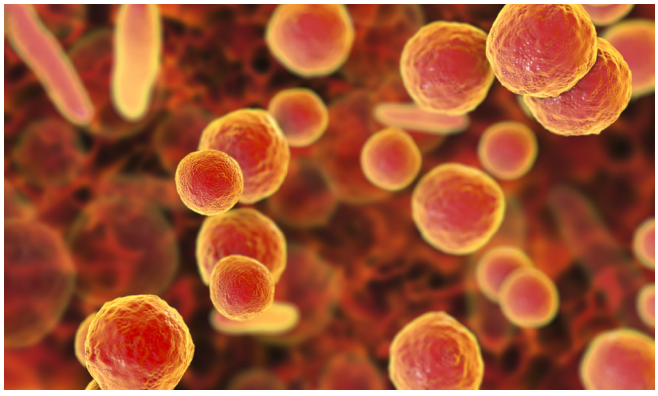
As pharmaceutical sales and manufacture continue to grow, so too does the demand for endotoxin testing reagents. Declining numbers of horseshoe crabs makes using LAL and TAL to test for bacterial endotoxins no longer sustainable. Furthermore, LAL and TAL are only produced in certain regions, and their availability is, as such, limited in some areas across the globe.[6]

To ensure the continued safety of our pharmaceuticals, it is clear that we need an alternative endotoxin test that is not only sustainable, but also widely available, and able to meet increasing endotoxin testing demands.

THE SHIFT TO SYNTHETIC REAGENTS

LAL tests rely on Factor C to act as a biosensor. In 1997, scientists from the National University of Singapore found that a recombinant Factor C (rFC) could be used to develop an animal-free endotoxin test, and Lonza produced the first commercial rFC product in 2010. Hyglos, now a bioMérieux company, has also developed and introduced an rFC now available in both conventional and rapid test formats.[6,8-10]

rFC is an exact synthetic copy of the Factor C found in lysate reagents. In rFC endotoxin tests, the rFC is added to a sample of the solution to be tested, and endotoxins present in the sample bind with the rFC, thus activating it. The rFC then reacts with a fluorogenic substrate, resulting in fluorescence which can be measured in order to determine the concentration of endotoxins in the sample. The results show that rFC BET provide not only high performance, but also reliable specificity, and consistency from a secure, sustainable, and animal-free source. [8-10]
rFC has been commercially available for fifteen years, but thus far, uptake has been slow, partly due to concerns regarding the efficacy of rFC tests in comparison to LAL. Endotoxins represent a serious health concern and their levels are highly regulated, and as such, pharmaceutical manufacturers have been understandably hesitant to change their testing methods, even though rFC may well be the better solution.[6]



Furthermore, regulatory bodies around the world rely on pharmacopeia to **harmonize processes and ensure uniform endotoxin testing procedures**. Until recently, rFC tests have not been included in the pharmacopeia, meaning that users have had to conduct their own validations.

As of July 2020, rFC enjoys compendial status in both the European Pharmacopoeia through a new general chapter 2.6.32 and the Chinese Pharmacopoeia updated in June of 2020.

The Japanese Pharmacopeia has also published a separate chapter draft on testing for bacterial endotoxins using rFC, and is expected to be considered compendial in April 2021. [11-13]

Lastly, in May 2020, the United States Pharmacopoeia (USP) published a Compendial Notice and Prospectus; reinforcing USP's commitment to the introduction of rFC into the official text of the USP-NF.

Kevin Williams is our resident endotoxin expert. Before joining bioMérieux, he worked in endotoxin testing at Eli Lilly for over thirty years, and he is also the author of several textbooks on endotoxin testing. William's believes that now is the time to start shifting towards rFC, and below, we have summarized his ten key reasons why.

No. 1

AN **ENVIRONMENTALLY RESPONSIBLE** CHOICE

While endotoxin tests using LAL and TAL reagents rely on capturing and bleeding horseshoe crabs, **rFC tests use synthetic reagents that are not dependent on animals**.

Choosing rFC instead of LAL or TAL may help the recovery of threatened and endangered populations. "Horseshoe crabs have been around for 400 million years, and now they are in danger of disappearing," says Williams.

Every year, **half a million horseshoe crabs from the Atlantic coastline are captured and bled**. Although the harvesting process doesn't kill the crabs, it does leave them disorientated and debilitated, with a reduced ability to spawn.

Estimates suggest that **15-30% of horseshoe crabs die in the weeks after the bleeding process, amounting to 130,000 crabs each year in North America.[6]**

— Kevin Williams

The impacts of the LAL harvesting process aren't limited to horseshoe crab populations.

"The ecosystem depends upon horseshoe crabs, and the fall in their numbers on Delaware bay is also threatening shorebirds who stop to fatten up on horseshoe crab eggs during migration," explains Williams, who highlights that the numbers of shorebirds across North America have declined by 70% since the 1970s.[6,14]

Moving to rFC for endotoxin testing is one way that companies can contribute to the conservation of threatened species, and also address the ever-growing concern for environmental issues among the public.

Estimates suggest that just by changing endotoxin tests conducted on water and other manufacturing materials to rFC, the demand for LAL from horseshoe crabs **would be reduced by 90%, saving approximately 100,000 crabs in North America alone**. A single 30L fermenter producing rFC replaces the bleeding of at least 6,000 horseshoe crabs, saving not only an increasingly endangered species, but also time and money.[6]

If crab numbers continue to decline - despite conservation efforts - we may not be able to meet the demand for endotoxin tests with LAL or TAL reagents alone. Moving to rFC would result in **a secure supply of testing reagents** that don't rely on the availability of horseshoe crabs. As such, if the horseshoe crab population collapses, endotoxin tests will still be available, and pharmaceutical production can continue without interruption.[15]

No. 2

CUTTING-EDGE RECOMBINANT PROTEINS

Across the past few decades, there has been something of a revolution in the pharmaceutical industry. Recombinant proteins are now used in a wide array of life-saving medicines and are becoming the new norm, with **around 400 recombinant therapies approved by the FDA**.[16,17]

This trend in itself fits neatly into a long line of similar changes within the industry. Indeed, the pharmaceutical industry has a rich history of moving from animal products to recombinant reagents — insulin for diabetics was once harvested from pigs or cows, and yet now, synthetic human insulin is produced using bacteria or yeast.[17]

The move to synthetic reagents is supported by European Union and US government directives aimed at reducing and replacing animals in medical testing and product manufacture. The reasons for this are financially as well as morally-driven; after all, moving from harvesting animal proteins to producing synthetic alternatives has several advantages, not least the fact that proteins obtained from animals can be inconsistent, and have the potential to be contaminated by disease-causing pathogens.

"Recombinant proteins produced using biotechnology are subject to more scientific rigor, resulting in high-quality reagents," explains Williams.[17-19]

"Pharmaceutical companies can ensure the quality and consistency of their rFC by choosing a reputable supplier with a global footprint," says Williams. There has been some concern about the quality of rFC as an alternative to LAL because its production is not FDA regulated, but as Williams points out, **LAL is only licensed by the FDA because it is a blood product, while rFC is not.**
The majority of reagents used in pharmaceutical manufacture are not blood products, and as a result, do not have FDA approval. In 2012 the FDA published guidance for the industry on endotoxin detection, stating that manufacturers may use rFC with method validation according to US Pharmacopeia chapters <85> and <1225>.
The FDA has recently approved the first drug to be tested for endotoxins using rFC rather than LAL, Emgality™ (galcanezumab), a migraine drug from Eli Lilly, showing their confidence in rFC tests.

"LAL is the exception rather than the rule in this case. To ensure their rFC quality, pharmaceutical manufacturers can audit the production process and conduct quality control tests, as they do with every other reagent they use."

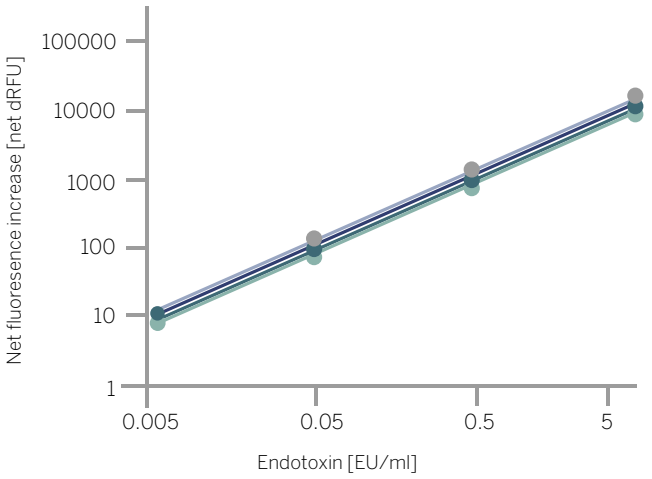
No. 3

AVAILABILITY, CONSISTENCY, AND WIDESPREAD SUPPLY

rFC can be produced consistently anywhere in the world, enabling global supplies to keep up with the demand for endotoxin testing. LAL, on the other hand, is regionally sourced and less widely available in some areas of the globe, most notably in Asia.

The synthetic production of rFC means that there is **less batch-to-batch variation than there is in LAL reagents** (see Figure 2), and rFC results are consistent even from one supplier to another.

In our tests on a range of endotoxin preparations, the correlation between rFC from two different manufacturers was 96.8%, while the correlation between LAL from two different manufacturers was only 93.6% (see Figure 3).



The high consistency of rFC means that **a product tested in China can be harmonized and compared with a test performed in the US**. A large study by a consortium of pharmaceutical manufacturers showed that when standardized procedures are used, consistent results can be obtained by rFC assays across many different laboratories.[21]

Figure 2: Endotoxin concentrations of standardized endotoxin preparations measured using rFC from different batches (ENDOZYME® II assay).

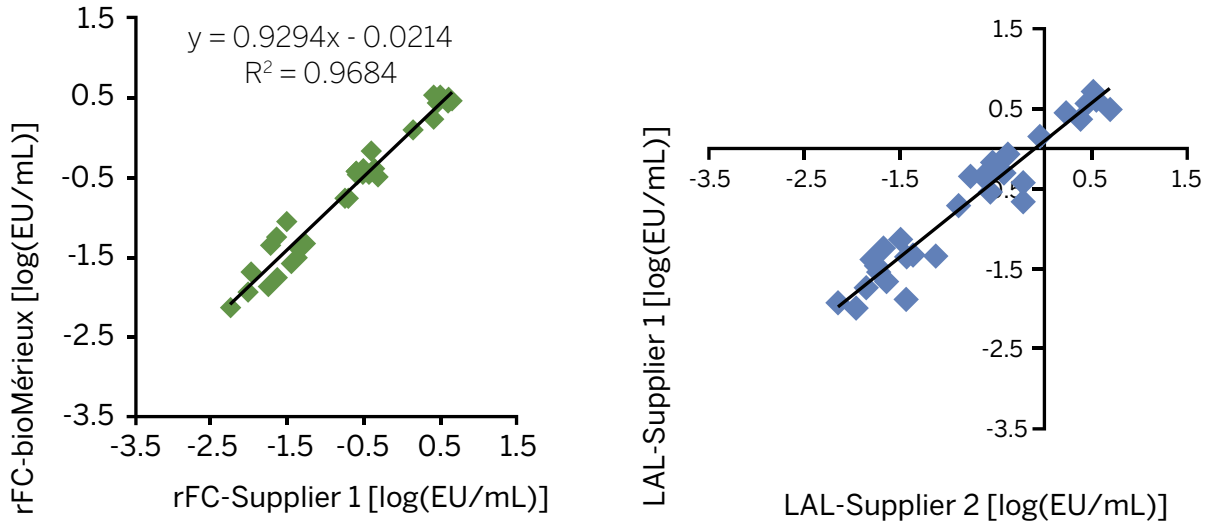


Figure 3: Endotoxin concentration results for standardized endotoxin preparations using rFC assays (left) and LAL assays (right) from two different suppliers.

No. 4

SIMPLIFIED AND FULLY CHARACTERIZED TEST MILIEU

As LAL is derived from natural sources, it contains **several by-stander proteins that could interfere with endotoxin testing and product proteins**. For example, LAL naturally contains serpin proteins that prevent clotting. These must be denatured or removed entirely before using LAL in endotoxin detection.[22]

In 1981, scientists discovered that LAL assays give **false-positive results** when exposed to β-glucans. This understandably caused quite a stir in the pharmaceutical industry, due to the fact that glucans are common constituents in manufacturing processes involving the breaking down of cellulosic fibers. β-glucans activate a protein in LAL called factor G, which then activates the clotting enzyme (see Figure 4), which results in a positive result from the LAL assay even if there are no endotoxins present.[22,23]

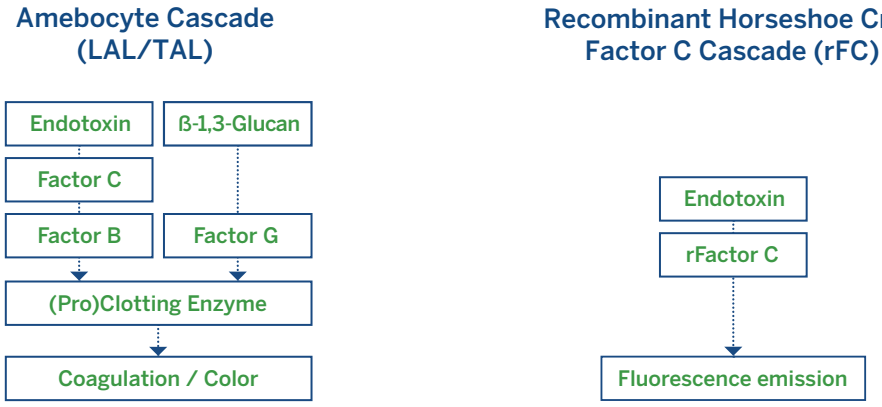


Figure 4: Diagram showing the cascade reaction in LAL assays and the alternative ß-glucan activation pathway that can cause false positives (left), compared to the rFC assay reaction (right).

Zwittergent is used in some LAL formulations to reduce the number of false positives by reducing sensitivity to β-glucans, yet this approach also reduces the LAL sensitivity to some bacterial endotoxins. Novitsky and Roslansky found that even a 0.01% change in Zwittergent content can result in a 16-fold difference in endotoxin concentration results; something which makes obtaining reliable results difficult.[22]

rFC reagents only contain the reagents needed for the test, with no by-stander or denatured proteins which may initiate alternative pathways. As a result, rFC assays specifically detect endotoxins and are unaffected by β-glucans and other molecules, and as such, there are far fewer false positives.

"If you are developing an endotoxin test with rFC and it doesn't work, you know exactly what is in your test milieu, which makes it easier to figure out what is going wrong" explains Williams. **"But if you are using LAL there could be interactions with serpins, glucans, zwittergent, or other substances in the solution."**

No. 5

SENSITIVE, SPECIFIC, AND RELIABLE ENDOTOXIN TESTING WITH rFC

"Previously, manufacturers have raised concerns about whether rFC assays perform as well as LAL assays," says Williams, "but their performance is quite comparable, with rFC offering slightly higher sensitivity and selectivity."

While LAL assays offer sensitivities in the region of 0.005 EU/mL, rFC sensitivity can be as high as 0.001 EU/mL. The higher sensitivity of rFC assays stems primarily from the use of fluorescence detection to determine the endotoxin concentration.

Fluorescence is one of the most sensitive and specific analytical methods, and is around **1000 times more specific than the absorbance techniques used by LAL assays.**

The excited fluorophore produced in rFC assays in the presence of an endotoxin emits light at a particular wavelength, allowing it to be precisely measured. Absorbance measurements, on the other hand, are frequently subject to interference. What's more, fluorescence measurements also provide accurate results for samples that are very dark, colored, or lack clarity, while absorbance measurements may struggle in the same conditions.[4]

Numerous studies have compared the performance of rFC and LAL endotoxin tests, and results consistently show that **the commercially available rFC tests perform at least as well as LAL tests, if not even better**. These studies demonstrate that rFC performs well across a wide variety of applications with a high limit of detection, even in the presence of potential inhibitors. Our studies also showed a high correlation (94.9-96.8%) between endotoxin measurements by rFC assays and LAL assays (see Figure 5).[21,24-30]

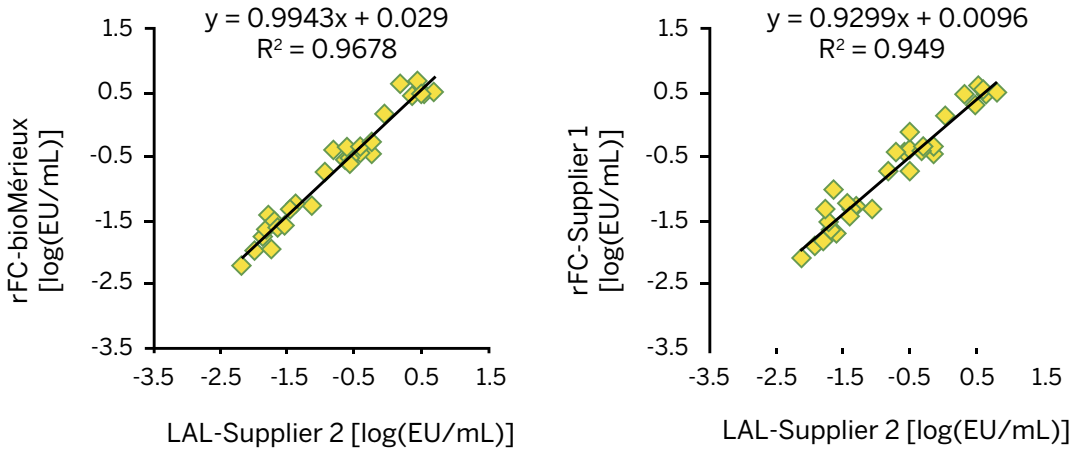


Figure 5: Comparison of endotoxin concentration results for standardized endotoxin preparations using rFC assays and LAL assays.

Inclusivity tests have consistently demonstrated that **rFC assays can detect all the endotoxins that are detected by LAL assays**. In studies carried out by the University Hospital of Munich, Germany, 200 endotoxin samples from different gram-negative bacteria were collected and tested, using an LAL assay and an rFC assay (ENDOZYME® rFC-bioMérieux). Both the LAL and rFC assays detected all 200 strains with a 94.3% correlation in quantification results.[31]

No. 6

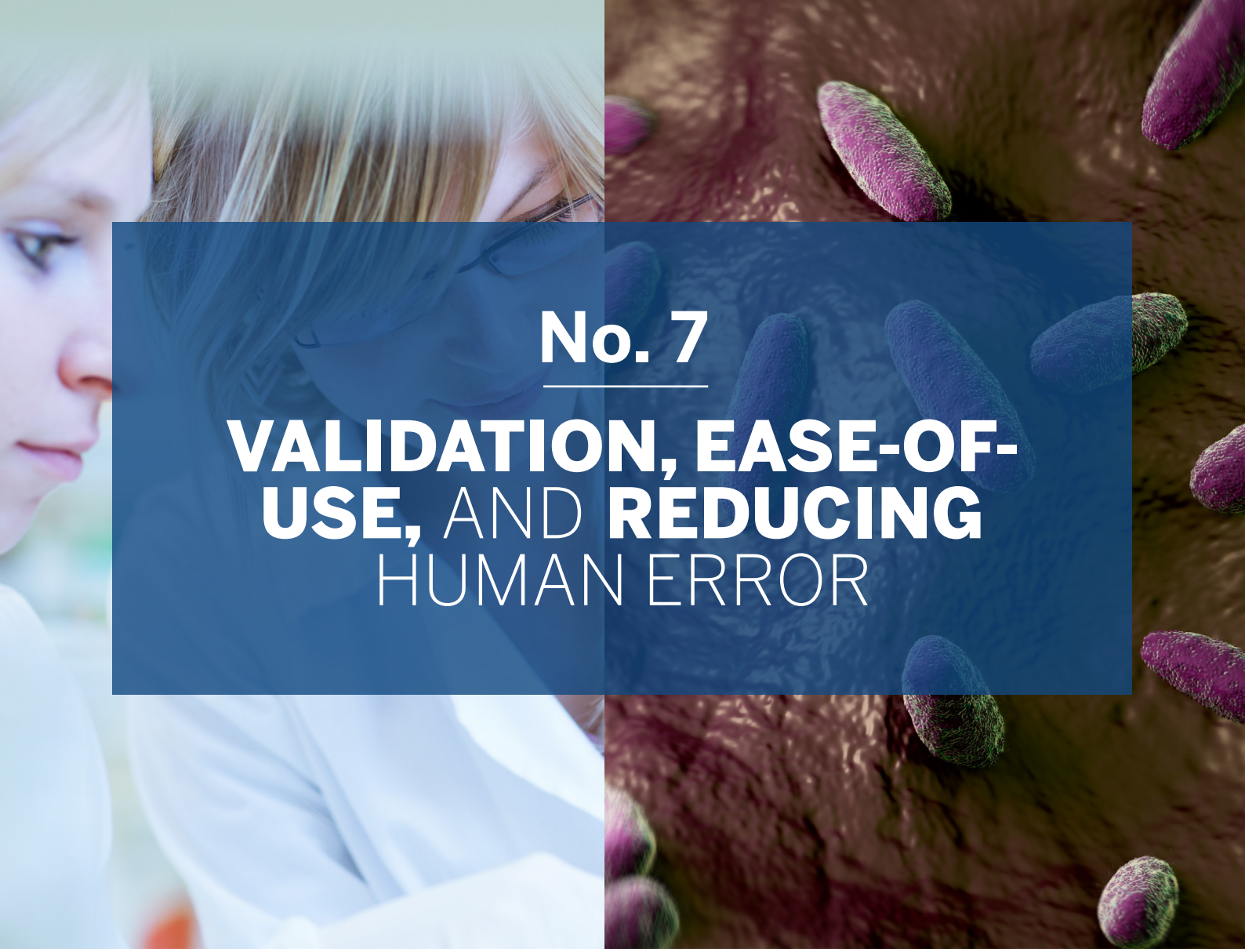
rFC: ACHIEVING COMPLIANCE WITH **COMPENDIA CRITERIA**

In order to comply with the International Pharmacopeia, **all endotoxin tests need to be verified as being free from additional interfering factors**. Once a final product is tested, and a valid endotoxin measurement has been obtained, a sample of the product is spiked with a known quantity of endotoxin. The endotoxin concentration is then measured again, and the mean recovery of the added endotoxin is calculated, leading to a result known as the percentage positive product control (%PPC). Ideally, the %PPC should be as close to 100% as possible, indicating that the assay detected all the additional endotoxin. For a final product to be released, **the FDA requires that %PPC is between 50% and 200%.**[32]

High batch-to-batch variations, as well as the potential for interference in LAL assays, means that relatively poor spike recovery can result. Many products and raw materials demonstrate better spike recovery with rFC compared with LAL, indicating lower levels of interference and better measurement consistency (see Table 1).

Category	Substance	Conc./ dilution	PPC recovery in ENDOZYME II GO [%]	PPC recovery in LAL1 [%]	PPC recovery in LAL2 [%]
Common excipients	Sodium citrate	1mM	112	161	92
		0.1mM	106	161	118
	Dextrose	5%	80	134	92
		0.5%	95	165	108
	NaCl	500mM	58	57	1
		50mM	92	135	84
	Polysorbate 20	0.02%	77	61	50
		0.002%	101	69	43
	PBS	1x	82	96	86
		1:10	100	142	110
Proteins	MAB-33	0.1 mg/mL	117	603	46
		0.01 mg/mL	108	190	92
	HSA	1 mg/mL	94	332	138
		0.1 mg/mL	111	188	76
Organic solvent	Ethanol	1%	99	135	94
		0.1%	114	174	104
Culture medium	IMDM	100%	35	214	55
		10%	91	262	130

Table 1: Interference (spike recovery) comparison of ENDOZYME® II GO recovery versus LAL tests.



No. 7

VALIDATION, EASE-OF-USE, AND REDUCING HUMAN ERROR

The current status of rFC assays as an 'alternative method' in the US Pharmacopeia, means that rFC methods require additional validation according to USP <1225> or International Conference on Harmonization (ICH) Q2Bs, to demonstrate that they are as effective or better than LAL. rFC assays must then be validated in the same way as LAL assays for suitability to particular products, as in accordance to USP <85>.

The validation process is not as complicated or time-consuming as people may believe, and indeed, it can be accomplished in a few days **so long as a well-structured protocol as provided by the suppliers is followed**.

Users can utilise existing literature as part of their validation when submitting products to the FDA for approval, as reported by Eli Lilly.[20,21]
rFC assays are easy to use, both as part of the validation process and during routine testing. Microplates pre-loaded with required standard curve reagents and positive product controls (PPC), **such as the GOPLATE™ in the ENDOZYME® II GO kit from bioMérieux**, are currently commercially available.

Using pre-prepared assays simplifies rFC testing and ensures reliable results every time. When users prepare their own assays, dilution, contamination, and mixing errors can result in failed tests and unreliable results. Prepared assays, therefore, simplify procedures and eliminate the risk of human error during preparation, resulting in higher precision and fewer invalid results.[33-35]

No. 8

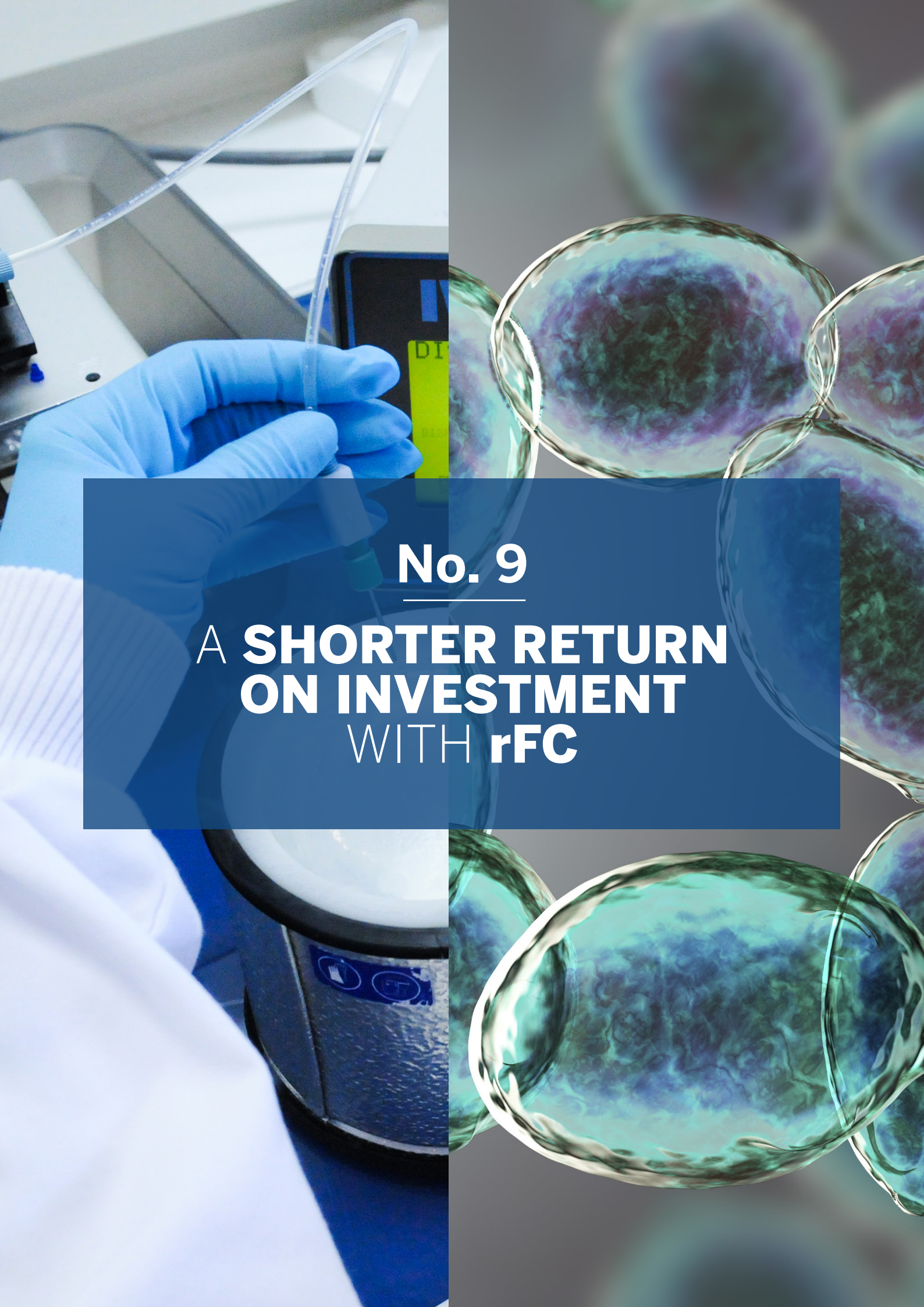
rFC: A **SWIFT** AND **EFFICIENT** SOLUTION

LAL assays often involve time-consuming workflows. **rFC tests can reduce the time required to obtain a result** and prevent repeat testing, further reducing time costs.[34]

Obtaining a result from an rFC assay typically takes **20-120 minutes**, depending not only on the type of assay, but also the sensitivity level required. Pre-loaded rFC plates remove the need to prepare standard solutions and dilutions, thus reducing hands-on time by around 50%.[34]

The ENDOZYME® II GOPLATE™ offers a swift workflow consisting of only three steps: adding water and sample to wells, preparing and adding the assay reagent, and running the assay through a fluorescence reader. The simplicity and direct nature of this system means it take just 20 minutes to obtain a result with 0.05 EU/mL sensitivity using an ENDOZYME II GOPLATE.[36]

While cartridge LAL assays have been developed to reduce preparation workloads, our study showed that they frequently produce more invalid results, increasing the need for repeat testing, and therefore eliminating any time benefits. Pre-loaded rFC plates, on the other hand, reduce invalid results, resulting in additional time savings. What's more, **rFC assays can also be combined with robotics and automation for maximum time efficiency and reliabiity (see point 10).**[34,36]



No. 9

A **SHORTER RETURN ON INVESTMENT** WITH **rFC**

Implementing any new technology requires investment, and rFC bacterial endotoxin tests are no exception to this rule. Although users will face costs associated with purchasing new readers and software, as well as training staff, and verifying procedures, **rFC assays cost less to run than LAL assays**, and as such, the time to see a return on any investment is, in fact, relatively short.

rFC assays offer savings in terms of analyst time, reagent costs, reduction in waste, and fewer repeat tests.

A pre-prepared rFC test kit **requires 50% less time** for preparation of calibration and positive product control solutions, saving approximately one hour of analyst time per kit compared with LAL tests.

What's more, it's important to point out that rFC assays **produce fewer invalid** results than LAL assays, thanks to higher levels of specificity, fewer false positives, and reduced batch-to-batch reagent variability. Fewer invalid results saves time and money spent on repeat tests (see Figure 6), thus making that initial investment go further.

rFC tests also **produce less waste** than LAL tests. The ENDOZYME® II GOPLATE™ is pre-filled, so only contains the amount of reagent needed for the test with no reagent going to waste. What's more, the plates have long-lasting expiry times, and are flexible, with no need to fill a full plate every time.

Overall, with their greater effiency, flexibility, and time-saving benefits, it is easy to see how using rFC quickly adds up to significant savings.[9,20]

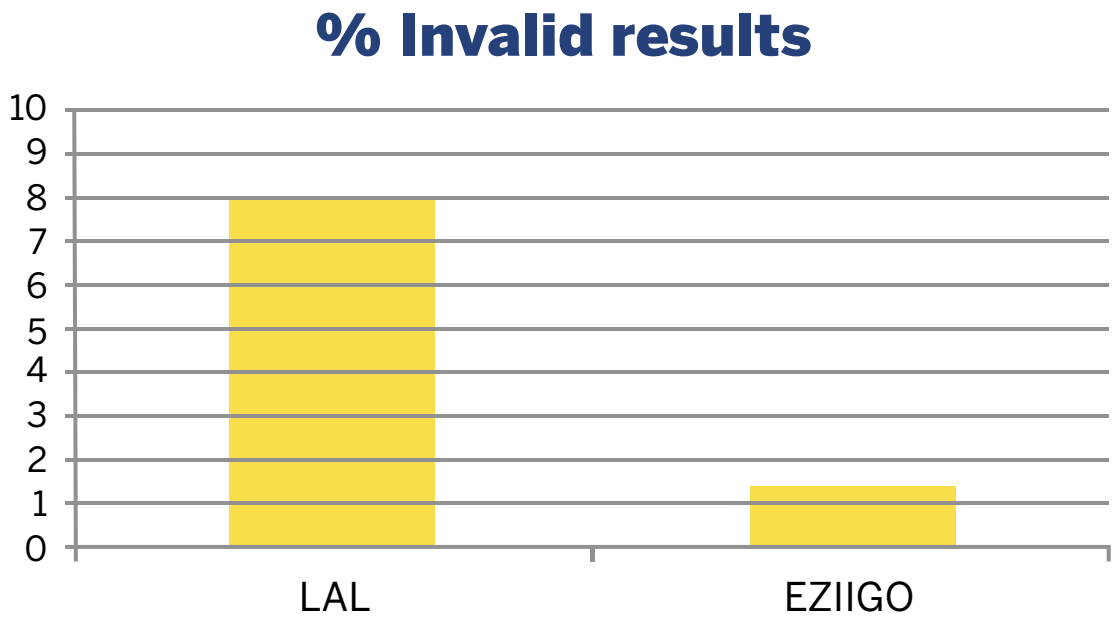


Figure 6: Comparison of the number of invalid results obtained with LAL tests compared with ENDOZYME® II GOPLATE.

Eli Lilly conducted a cost analysis and calculated their expected return on investment before implementing rFC, with their findings demonstrating that it was indeed a cost-effective option. For companies wishing to calculate the return on investment time, a tool is available from bioMérieux that weighs the costs of instrumentation and validation against savings from reagent costs, staff time, waste reduction, and the reduction in the number of invalid results.[9,20]

No. 10

FULFILLING **DATA INTEGRITY NEEDS** WITH **SMART AUTOMATION**

In recent years, the pharmaceutical industry has seen dramatic shifts towards widespread automation, with one of the principal aims being the improvement of process control and data integrity.

"rFC tests are easier to automate than LAL assays," explains Williams."The rFC reagent is stable for several hours after mixing, so it can sit on the bench in light at room temperature without deteriorating." To help facilitate automation, we have also developed simplified software to make automated endotoxin tests more accessible and more efficient than ever.

We developed a prototype semi-automated workflow for bacterial endotoxin testing using a pipetting robot and ENDOZYME® II GOPLATE™. The pipetting robot transfers liquid from sample tubes into the microplate, reducing handling time and eliminating pipetting errors. We found that a full plate of 20 samples in duplicate was ready for detection in 13 minutes, with automation also significantly increasing the accuracy and precision of the results (see Figure 7).

Recently, the ENDOZYME II GOPLATE has also been implemented in a high-throughput robotic system by the Novartis Institutes for Biomedical Research in Switzerland. The system used three incubators, a fluorescence reader, and an automated liquid handling workstation to run three plates in parallel with one reader, delivering the results of 60 samples in just 2.5 hours.

rFC testing is well suited to automated processes which provide efficient and error-resistant high throughput testing.[36]

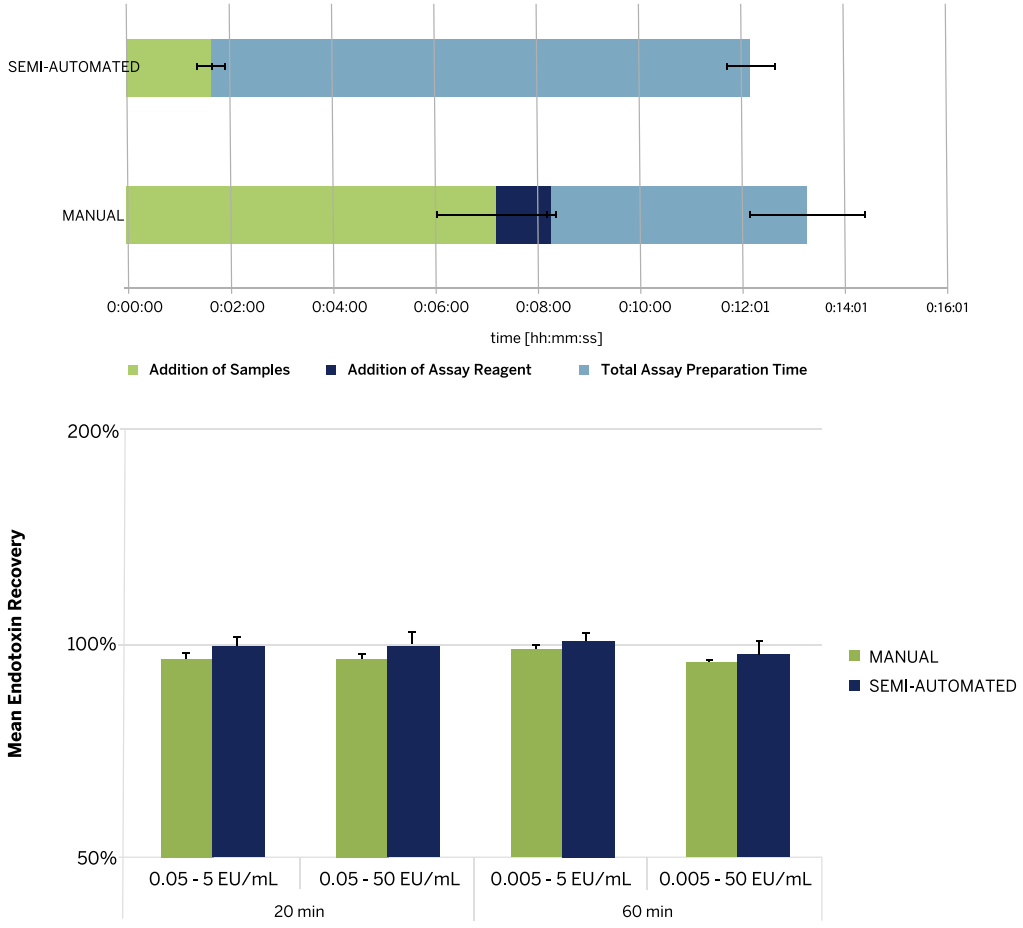


Figure 7: Total preparation times and hands-on times for performing ENDOZYME II GO with semi-automated and manual workflow (left), %PPC recovery (0.5 EU/mL) for manual and semi-automated testing using ENDOZYME II GO.

Endotoxin testing is a vital part of the pharmaceutical manufacturing industry, and there is no doubt that accurate, reliable, and sustainable endotoxin testing procedures are critical for ensuring the safety of injectables and medical devices.

For forty years, LAL has ensured the safety of our pharmaceutical and medical products, but today the time has come to move on to a better and more sustainable option. rFC assays represent the next generation of BET solutions which offer a combination of high performance, security, and cost-effectiveness, making them the superior choice compared with LAL assays. As regulations and pharmacopeia are beginning to reflect the suitability of rFC tests, we are beginning to see pharmaceutical manufacturers making the switch and benefiting accordingly.

Established validation procedures and past FDA approvals mean that achieving the approval of drugs using rFC testing is highly straightforward, and the latest testing solutions mean that using rFC tests has become simpler than ever. The flexibility, speed, and efficiency of rFC endotoxin tests makes them ideal for both in-process control of water and raw materials, and product release testing, thus further highlighting the importance of this groundbreaking next step for endotoxin testing.

1. Alexander C, Rietschel ET. **Bacterial lipopolysaccharides and innate immunity.** *J Endotoxin Res.* 2001;7(3):167-202.

2. Williams K. *Endotoxin Detection and Control in Pharma, Limulus, and Mammalian Systems.* Springer International Publishing; 2019.

3. US Food & Drug Administration. **Bacterial Endotoxins/Pyrogens.** https://www.fda.gov/inspections-compliance-enforcement-and-criminal-investigations/inspection-technical-guides/bacterial-endotoxinspyrogens. Content current as of: November 17, 2014. Accessed April 16, 2020.

4. McCullough KZ. *The Bacterial Endotoxins Test: A Practical Approach.* Bethesda, MD.: Davis Healthcare International Publishing, LLC; 2011.

5. Iwanaga S. **Biochemical principle of Limulus test for detecting bacterial endotoxins.** *Proc Jpn Acad Ser B Phys Biol Sci.* 2007;83(4):110–119.

6. Maloney T, Phelan R, Simmons N. **Saving the horseshoe crab: A synthetic alternative to horseshoe crab blood for endotoxin detection.** *PLoS Biol.* 2018;16(10):e2006607.

7. Smith DR, Beekey MA, Brockmann HJ, King TL, Millard MJ, Zaldívar-Rae JA. **Limulus polyphemus.** *The IUCN Red List of Threatened Species.* 2016:e.T11987A80159830.

8. Ding JL, Navas MA 3rd, Ho B. **Molecular cloning and sequence analysis of Factor C cDNA from the Singapore horseshoe crab, *Carcinoscorpius rotundicauda*.** *Mol Mar Biol Biotechnol.* 1995;4(1):90–103.

9. Bolden J, Smith K. **Application of Recombinant Factor C reagent for the detection of bacterial endotoxins in pharmaceutical products.** *J Pharm Sci Technol.* 2017;(5):405–412.

10. Mizumura H, Ogura N, Aketagawa J, et al. **Genetic engineering approach to develop next-generation reagents for endotoxin quantification.** *Innate Immun.* 2017;23(2):136–146.

11. Council of Europe. **New general chapter on test for bacterial endotoxins using recombinant factor C. Give your feedback.** January 9, 2019. https://www.edqm.eu/en/news/new-general-chapter-test-bacterial-endotoxins-using-recombinant-factor-c-give-your-feedback. Accessed April 16, 2020.

12. Japanese Pharmacopoeia. **Draft chapter of alternative endotoxin testing.** October 16, 2019. http://www.pmda.go.jp/files/000231653.pdf. Accessed April 16, 2020.

13. ECA Academy. **Japanese Pharmacopoeia - draft chapter of alternative endotoxin testing.** https://www.gmp-compliance.org/gmp-news/japanese-pharmacopoeia-draft-chapter-of-alternative-endotoxin-testing. October 16, 2019. Accessed April 16, 2020.

14. Munro M. **What's killing the world's shorebirds?** *Nature.* 2017;541(7635):16–20.

15. Carmichael RH, Botton ML, Shin PKS, Cheung SG. *Changing Global Perspectives on Horseshoe Crab Biology, Conservation and Management.* Springer Nature. 2015.

16. US Food & Drug Administration. **Fact sheet: FDA at a glance.** https://www.fda.gov/about-fda/fda-basics/fact-sheet-fda-glance. Content current as of October 18, 2019. Accessed April 16, 2020.

17. Pavlou AK, Reichert JM. **Recombinant protein therapeutics—success rates, market trends and values to 2010.** *Nat Biotechnol.* 2004;22(12):1513-1519.

18. **Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.** http://data.europa.eu/eli/dir/2010/63/oj. Posted October 20, 2010. Accessed April 16, 2020.

19.US Environmental Protection Agency. **Administrator Wheeler signs memo to reduce animal testing, awards $4.25 million to advance research on alternative methods to animal testing.** Chemical Safety and Pollution Prevention(OCSPP). https://www.epa.gov/newsreleases/administrator-wheeler-signs-memo-reduce-animal-testing-awards-425-million-advance. Released September 10, 2019. Accessed April 16, 2020.

20. bioMérieux Webinar. **Endotoxin testing using Recombinant horseshoe crab Factor C (RFC).** Available at: https://go.biomerieux.com/Endotoxin-testing-Webinar. Accessed April 16, 2020.

21. Bolden J, Knight M, Stockman S, Omokoko B. **Results of a harmonized endotoxin recovery study protocol evaluation by 14 BioPhorum Operations Group (BPOG) member companies.** *Biologicals.* 2017;48:74–81.

22. Roslansky PF, Novitsky TJ. **Sensitivity of Limulus amebocyte lysate (LAL) to LAL-reactive glucans.** *J Clin Microbiol.* 1991;29(11):2477-2483.

23. Cooper JF, Weary ME, Jordan FT. **The impact of non-endotoxin LAL-reactive materials on Limulus amebocyte lysate analyses.** *J Pharm Sci Technol.* 1997;51(1):2–6.

24. Grallert H, Leopoldseder S, Schuett M, Kurze P, Buchberger B. **EndoLISA®: a novel and reliable method for endotoxin detection.** Hyglos GmbH, Bernfied Germany.

25. Kikuchi Y, et al. Collaborative study on the bacterial endotoxins test using recombinant factor C-based procedure for detection of lipopolysaccharides. *Pharm Med Device Reg Sci.* 2017;48(4):252-260.

26. Abate W, Sattar A, Liu J, Conway M, Jackson S. **Evaluation of recombinant factor C assay for the detection of divergent lipopolysaccharide structural species and comparison with Limulus amoebocyte lysate-based assays and a human monocyte activity assay.** *J Med Microbiol.* 2017:12;66(7):888-897.

27. Reich J, Heed K, Grallert H. **Detection of naturally occurring bacterial endotoxins in water samples.** *Eur Pharm Rev.* 2014;19(6):67-68.

28. Chen L, Mozier N. **Comparison of Limulus amebocyte lysate test methods for endotoxin measurement in protein solutions.** *J Pharm Biomed Anal.* 2013;80:180-185.

29. Thorne PS, Perry SS, Saito R, et al. **Evaluation of the Limulus amebocyte lysate and recombinant factor C assays for assessment of airborne endotoxin.** *Appl Environ Microbiol.* 2010;76(15):4988-4995.

30. Loverock B, Simon B, Burgenson A, Baines A. **A recombinant factor C procedure for the detection of Gram-negative bacterial endotoxin.** 2010;36(1):321-329.

31. Publication Wieser et al. in preparation

32. World Health Organization. **Final text for revision of *The International Pharmacopoeia.*** https://www.who.int/medicines/publications/pharmacopoeia/Bacterial-endotoxins_QAS11-452_FINAL_July12.pdf. July 2012. Accessed April 16, 2020.

33. https://biomerieuxdirect.com/industry/c/ENDOZYME®-II-GO/p/890031

34. Schneider P, Vogl M, Uhlig T, Grallert H, Devulder G. **A time-saving Recombinant Factor C (rFC) endotoxin test including a novel microplate pre-coated with control standard endotoxin (CSE) concentrations and positive product controls.** Presented at PDA Annual Meeting 2018. Available at: https://www.biomerieux-industry.com/sites/default/files/2019-06/POSTER_PDA_2018_-_ENDOZIME_II.pdf. Accessed April 16, 2020.

35. Marine M. 'Endotoxin testing in pharmaceutical water – a method comparison study'

36.Schneider P, Vogl M, Uhlig T, Grallert H, Devulder G. **Evaluation of a semi-automated endotoxin testing workflow using a Recombinant Factor C (rFC) assay and an automated pipetting robot.** Presented at PDA Europe BioManufacturing Conference 2019. Available at: https://www.biomerieux-industry.com/pharma-healthcare/resources/scientific-posters/2019-09-10-evaluation-semi-automated-endotoxin. Accessed April 16, 2020.