
Confidence for adopting the rFC method in your lab

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Pharma & Biotech

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This article explores the history of the LAL assay, why LAL is regulated, and the regulatory status of the recombinant Factor C assay. The content is intended to serve as fact-based reference material for those companies who would like to consider adopting the rFC method in their labs.

Recently, articles have been published questioning the use of recombinant Factor C Assays to detect endotoxin contamination in parenterally administered pharmaceuticals or implanted medical devices. There seems to be some confusion regarding the history of the development of the LAL assay, the development of the rFC assay, and its regulatory status.

Why is LAL Regulated?

It may surprise many to know that LAL is not regulated for what it does, but rather where the reagent comes from. In the early stages of LAL development, The United States Food and Drug Administration (FDA) was very interested in this new assay, and began extensive research into its potential use to detect endotoxin in pharmaceutical products. Seligman, Hochstein, and Cooper at FDA were instrumental in developing this assay for use. Because of its animal origin nature, and inherent variability of such products, FDA decided to regulate the product if used as a pyrogenicity test for pharmaceutical products. In 1973, in a Federal Register Notice (Federal Register, 1973), FDA announced that LAL was a biologic that would be regulated by FDA under Section 351 (h) of the Public Health Service Act, which stated:

(i) In this section, the term "biological product" means a virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, or analogous product, or arsphenamine *or derivative* of arsphenamine (or any other trivalent organic arsenic compound), applicable to the *prevention*, treatment, or cure of a disease or condition of human beings.

Biologics are a subset of drugs, and the definition of a drug from section

201 of the US Food, Drug, and Cosmetic Act (FD&C Act) is:

- A substance recognized by an official pharmacopoeia or formulary
- A substance intended for use in the diagnosis, cure, mitigation, treatment, or *prevention* of disease
- A substance (other than food) intended to affect the structure or any function of the body
- A substance intended for use as a component of a medicine but not a device or a component, part or accessory of a device
- Biological products are included within this definition and are generally covered by the same laws and regulations, but differences exist regarding their manufacturing processes (chemical process versus biological process)

LAL did not enter the United States Pharmacopeia (USP) until Volume XX 1980, and was not defined in this Compendia until then. LAL is not injected into the body, or applied, to act in a chemical means to affect the structure of the body. It does not diagnose, cure, treat, or mitigate any disease. Therefore, the best, but tenuous, fit was that it was meant to prevent contaminated drugs from entering into commerce, as the Pyrogen Test using rabbits did. Many assays do just that as final product release assays, but without the need for FDA regulatory oversight through licensing of the assay itself.

In 2002, Cambrex BioScience (now Lonza) filed a Request for Designation (RFD) with FDA to determine the regulatory status of its new rFC product, in order to obtain regulatory approval by either CBER or CDRH. Cambrex understood that the rFC product did not originate from

horseshoe crab blood, so this tenuous association could not be used for CBER to regulate the product. The FDA responded to Cambrex's RFD [US Food & Drug Administration 2003]:

The RFD covers the PyroGene™ Assay an *in vitro* diagnostic (IVD) test which, according to the RFD, is intended to test other products for endotoxin contamination prior to the other products' final release. The RFD states that the PyroGene™ Assay is not intended to detect endotoxemia in man or animals.

According to the RFD, the PyroGene™ Assay works in a manner similar to the Limulus Amebocyte Lysate (LAL) endotoxin test, except that the LAL test relies on Factor C derived from horseshoe crabs whereas PyroGene™ Assay uses a recombinant Factor C. All other components of the PyroGene™ Assay are synthetically produced. The RFD states that the endotoxin detection limit and linear assay range of the PyroGene™ rFC Assay are comparable to currently marketed LAL tests. Currently marketed LAL tests are reviewed and regulated by FDA's Center for Biologics Evaluation and Research (CBER). The RFD requests that the PyroGene™ Assay be assigned to the Center for Devices and Radiological Health (CDRH) since the PyroGene™ Assay does not make use of any live animals, and CDRH has developed relevant expertise through its review of other *in vitro* diagnostic products.

*CBER has regulated LAL tests for many years because of the possibility of variation inherent in animal-derived products, and because these products are intended to test blood and/or blood products for endotoxin contamination. CDRH regulates *in vitro* diagnostics intended for use in clinical diagnosis and patient management.*

We have carefully considered the information in the RFD and discussed the issues raised with staff in both Centers. Because the PyroGene™ Assay is used to test for endotoxin contamination in other products and not man or animals, is not intended to qualify blood or blood products, and is not intended for use in patient management, we conclude that it does not require premarket approval. Accordingly, *no premarket submission to either CBER or CDRH will be required.*

It wasn't that rFC didn't perform well enough to be regulated, it didn't need to be regulated by either CBER or CDRH because it was not an animal-derived product with its inherent variability. FDA clearly stated in its response to Cambrex's RFD that a premarket submission was not necessary for use in testing pharmaceutical products for release into commerce.

GMP Manufacturing of rFC Reagents

It is important when sourcing any material from a vendor that the customer understands the manufacturing conditions, and the quality systems under which the product is manufactured. A reputable vendor with a long track record of compliance is a strong indicator that the material is of high quality.

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Lonza's PyroGene™ rFC Endotoxin Detection Assay is made in the same FDA-licensed facility as our traditional FDA-licensed LAL products (PYROGENT™ Gel Clot, PYROGENT-5000™ Kinetic Turbidimetric, QCL-1000™ Endpoint Chromogenic, and Kinetic-QCL™ Kinetic Chromogenic Assays), using the same highly trained personnel, under the same quality system as those FDA-licensed LAL products. In addition to biannual inspections by FDA, for those facilities, quality systems, and manufacturing processes, our classical LAL, and now PyroGene™ rFC Assay, customers audit Lonza many times per year.

Furthermore, Lonza filed a Master File with FDA/CBER in 2008 (BBMF-13800), which contains the same type of Chemistry, Manufacturing, and Controls (CMC) information, including the chemistry of the reaction and reaction mixtures, the complete manufacturing process, and all of the QC information for batch release, that would be submitted to FDA in a BLA for LAL. The information contains a list of all the raw materials used in production, including the vendor names and raw material specifications. In addition to the complete CMC section, the Master File contains a complete facilities section, and a complete product validation section. The validation section contains all of the raw data and final reports for the assay validation, including the complete assay validation required by FDA (US Food and Drug Administration 2012) under USP <1225>, and published in the USP Pharmacopeial Forum in the Jan/FEB 2010 PF issue. Any change to any part of the Master File is reported to FDA/CBER in the Annual Report to the Master File. This Master File was cross-referenced by several pharmaceutical companies in preparing regulatory filings with FDA. The first product approved by FDA using the PyroGene™ rFC Assay as the final endotoxin release test was Eli Lilly's Emgality™. There are other customer's products in the pipeline for approval by FDA in the near future.

Lonza also published a Stimulus to Revision article in the USP Pharmaceutical Forum in the Jan/Feb 2010 issue (Loverock, et al. 2010). This document detailed Lonza's validation efforts as per FDA and USP requirements for an Alternative Assay (USP 2012) (US Food and Drug Administration 2012). This validation used ten representative pharmaceutical products across the pharmaceutical spectrum of products, which were tested in six different international facilities. The results demonstrated that Lonza's rFC product performed as well as the classical and compendial Kinetic-QCL™ Assay from Lonza. All of the final reports and the raw data of this study are contained in Lonza's FDA/CBER Master File (BBMF-13800).

Regulatory Acceptance of rFC Assays

FDA stated the following in their 2012 Q&A Guidance regarding the use of an Alternative Assay (such as rFC) (US Food and Drug Administration 2012):

May a firm use alternative assays to those in the USP for a compendial article?

Yes, firms may use alternative methods and/or procedures if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data

reduction, and in other special circumstances. Such alternative procedures and methods should be validated as described in the USP General Chapter <1225>, Validation of Compendial Procedures, (USP 2012) (USP 2011) and should be shown to achieve equivalent or better results.

(1) Recombinant Horseshoe Crab Factor C Assay

If a manufacturer chooses to use a recombinant factor C-based assay, the method validation should be in accordance with the requirements of USP Chapter <85>, Bacterial Endotoxins Test, as described in the section for Photometric Quantitative Techniques, and USP Chapter <1225>, Validation of Compendial Procedures. (USP 2011)

As stated above, the rFC assay has been validated for use as an alternative assay dozens of times, and FDA has reviewed the Master File and customer data in customer's regulatory filings.

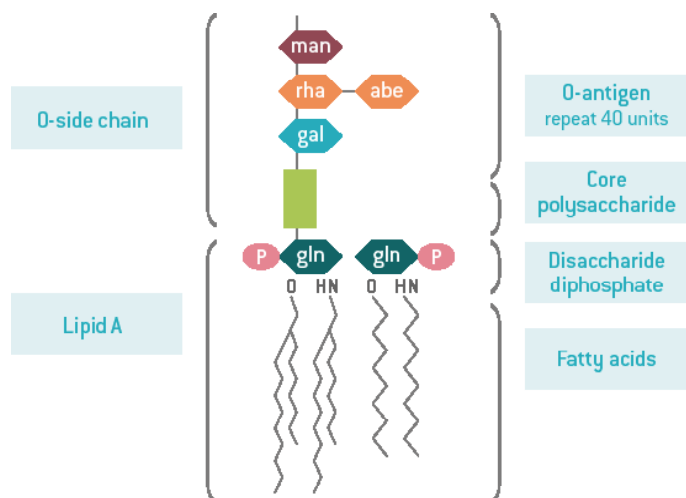
Endotoxin Basics

Endotoxin is a ubiquitous substance found in the environment. It is in our gut, in the water we drink, and on the surfaces we touch. Every animal that walks this earth, flies its skies, or swims its waters all produce endotoxin from flora contained in their gut. Endotoxin naturally occurs in water and soil as a part of the Gram-negative bacteria that inhabit those environments. We are all fine and healthy as long as the endotoxin stays where it belongs, outside of our bloodstreams or cerebrospinal fluid. It is when this barrier is breached, and endotoxin enters these fluids, that we have a response to them.

Endotoxin is found in the outer membrane layer of Gram-negative organisms. It is composed of three layers; the highly variable O-antigen, the core polysaccharide, and Lipid A. Lipid A is composed of a diphosphorylated glutamine disaccharide. Fatty acids are attached to the oxygen and nitrogen of the disaccharide, and could contain anywhere from four to seven fatty acids, depending on the species of bacteria. It is the Lipid A portion that conveys pyrogenicity to the lipopolysaccharide in its interaction with TLR-4 (amongst others). This same region interacts with the sushi domains of Factor C in the Limulus Amebocyte Lysate enzymatic cascade. These sushi domain sequences have been around for hundreds of millions of years, and are the basis of the innate immune system of the horseshoe crabs. These sushi domains are highly conserved across the family Limulidae.

Comparisons to LAL regarding Various Endotoxin Species

It would be nearly impossible to test every species and strain of Gram-negative endotoxin as one writer seems to suggest be done. Bergey's Manual of Determinative Bacteriology contains thousands of such organisms, and those are only the ones currently known. No LAL reagent manufacturer has done so. The significant differences in the Lipid A structure are what is of interest, and what is found in the current literature. These differences are the number of acyl chains, and the



Lipid A

The pyrogenic feature of LPS is Lipid A. All Lipid A portions contain two glucosamines bonded by a 1-6 linkage. The diglucosamines are phosphorylated at the C1 and C4 positions, forming diester bonds (Pearson 1985).

Acyl groups of the Lipid A are attached to either the O or N of the diglucosamines. The acylation may contain anywhere from four to seven acyl groups, with hexa-acylation being the most common in the Enterobacteriaceae, and are generally the most potent. The acyl chains vary in length between species. It is the acyl groups of that confer the hydrophobicity to Lipid A, causing them to bury themselves in the lipid bilayer of the cell membrane.

length of those chains. Lonza published its initial data in a Stimulus to Revision article USP's Official Journal Pharmacopeial Forum (Loverock, et al. 2010). In that article, we presented data regarding the detection of *Pseudomonas aeruginosa*, *Salmonella minnesota*, *E. coli* O55:B5, and *E. coli* O113 (USP EC-6 RSE) using Lonza's kinetic chromogenic, kinetic turbidimetric, and rFC assays. There were no significant differences in detection between the rFC and kinetic chromogenic assays for any of the endotoxin species, while the kinetic turbidimetric assays detected slightly higher endotoxin activity for the *P. aeruginosa* and *S. minnesota* endotoxins. However, the rFC data fell between both the kinetic LAL reagents.

Others have compared rFC performance with compendial LAL products. Bolden and Smith (Bolden and Smith 2017) published their findings in their comparison of rFC-based methods and LAL using contamination from several different organisms. Abate et al. compared Lonza's PyroGene™ rFC Assay to two kinetic chromogenic products and the Monocyte Activation Test (MAT) in detecting endotoxin from several different Gram-negative species (Abate, et al. 2017). These species represented the different acylation differences in Lipid A, and included quatra-acyl, penta-acyl, hexa-acyl, and hepta-acyl species. The rFC assay was able to detect and quantify these endotoxins as well as the LAL assays. The exception is *F. tularensis*. This endotoxin was detected by both Lonza's PyroGene™ and KQCL Assays, but not the other manufacturer's compendial product. The difficulty in detection is linked to this organism's ability to evade detection and cause disease in humans and animals.

To request a Validation Protocol, visit: www.lonza.com/pyrogene

In Summary

rFC reagents perform as well as LAL reagents in detecting endotoxin of various species in different matrices in our and our customer's hands. There was no significant difference between rFC and kinetic chromogenic reagents. FDA has determined that rFC reagents are acceptable for final product release, provided they are validated against the criteria found in USP <1225>.

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