PyroGene™ recombinant Factor C Assay
for endotoxin detection

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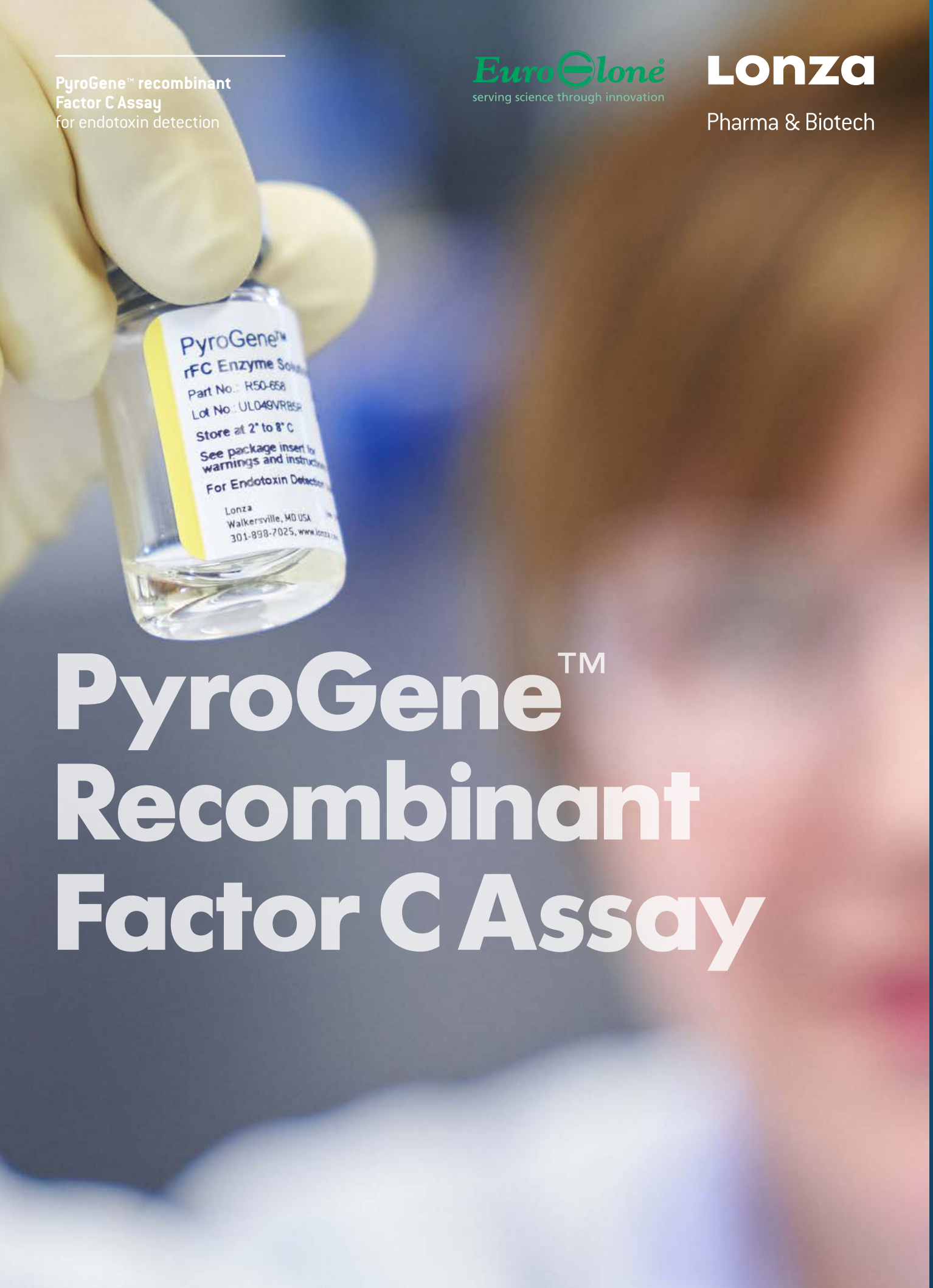


PyroGene™ Recombinant Factor C Assay

The LAL test was commercially introduced in the 1970s for the detection of endotoxin in biological products, parenterals and medical devices.

LAL is derived from the blood cells, amebocytes, of the horseshoe crab, *Limulus polyphemus*. The first LAL test method developed was the Gel Clot LAL (PYROGENT™ Assay) which provides a simple qualitative detection of endotoxin. Subsequent advances were made that lead to the development of more sensitive and quantitative test methods such as the turbidimetric (PYROGENT™-5000) and chromogenic (QCL-1000™, Kinetic-QCL™) assays. All of these test methodologies share the same basic mechanism of action, the LAL clotting cascade shown in Table 1.

PyroGene™ rFC Assay

The PyroGene™ Recombinant Factor C Assay is the evolution of the LAL test. Combining 21st century technology with the horseshoe crab's endotoxin sensitive protein, Lonza has developed an equivalent, reliable and sustainable endotoxin detection method for the future.

Factor C, the first component in the horseshoe crab clotting cascade, is activated by endotoxin. Lonza scientists have developed a recombinant form of Factor C, Recombinant Factor C (rFC), that is activated by endotoxin binding. The active enzyme then cleaves a synthetic substrate, resulting in the generation of a fluorogenic compound.

The PyroGene™ rFC Assay works through a single enzymatic step compared to the multiple step enzymatic process necessary for LAL assays (Figure 1).

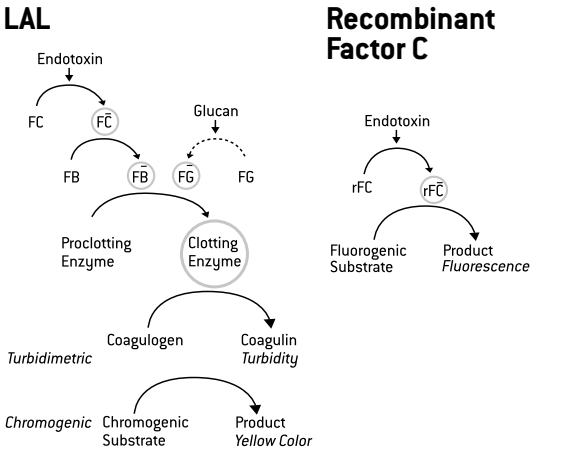
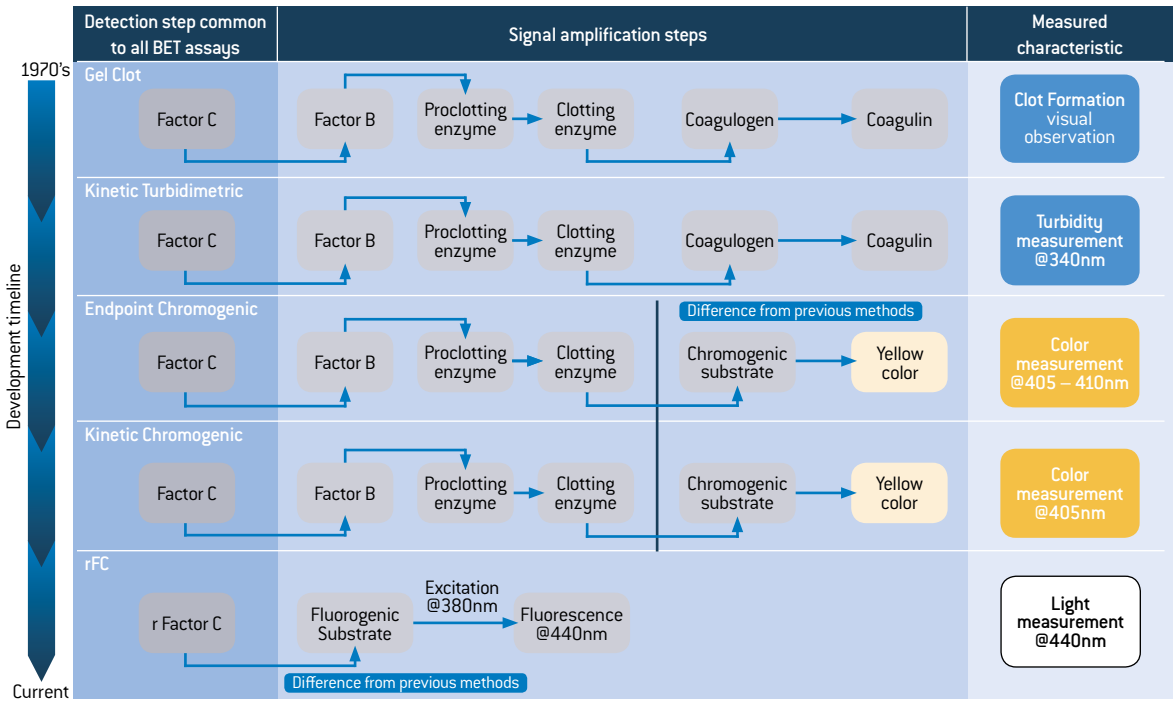


Figure 1: The traditional LAL clotting cascade can be triggered by endotoxin or glucans compared to the one-step rFC reaction, not triggered by glucans.



Comparison of amplification methods in bacterial endotoxin detection assays

Table 1: Comparison of the amplification steps required for LAL-based assays versus the rFC method. The rFC method is a more direct detection methodology than LAL-based assays, only requiring the binding of endotoxin to activate cleavage of the fluorogenic substrate to produce a quantifiable signal.

Due to its recombinant origin, the PyroGene™ rFC Assay does not use horseshoe crab blood as the raw material. Therefore, it offers a reliable, sustainable method for endotoxin detection.

The PyroGene™ rFC Assay includes the rFC enzyme cloned from the horseshoe crab. The working reagent is produced by mixing the liquid format rFC enzyme, buffer, and fluorogenic substrate, thus eliminating the lysate reconstitution step common to all the LAL assay methods. This allows for more flexible use of the kit components as one only needs to prepare the reagent mix for the actual number of tests to be run.

In the PyroGene™ rFC Assay, activated Factor C directly cleaves a fluorogenic substrate, producing a signal that is read using a standard fluorescent plate reader. Due to the high dynamic range of the fluorescent signal, the PyroGene™ rFC Assay delivers a quantitative range of 0.005 – 5.0 EU/mL in a single step, with better resolution than conventional kinetic LAL assays.

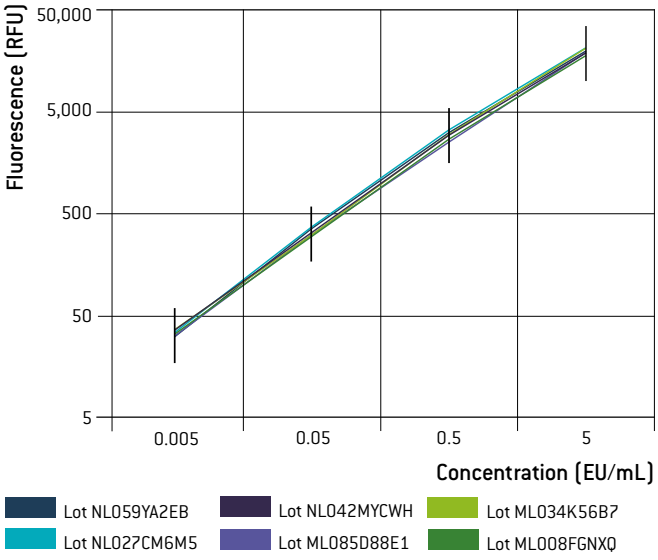
The reaction is run in a 96-well microplate and is measured at time zero and after a one-hour incubation in a fluorescent microplate reader using excitation/emission wavelengths of 380/440 nm. In the presence of endotoxin, activated rFC will cleave the fluorogenic substrate, causing the solution to fluoresce. The log net fluorescence is proportional to the log endotoxin concentration and is linear in the 0.005 – 5.0 EU/mL range. This single step reaction provides better resolution than conventional LAL assays.

Benefits over conventional LAL assays

- Endotoxin specific, recombinant technology eliminates false-positive glucan reactions
- Predictable, reliable lot-to-lot assay performance
- Sustainable resource – no animals used in testing or reagent manufacturing
- Statistically more robust
- Provides greater security of supply
- Ease-of-use due to liquid reagent format – no reconstitution of lyophilized components, working solution volume corresponds to number of tests to be run
- Better resolution than conventional LAL methods
- Sensitivity range from 0.005 - 5.0 EU/mL
- Provides a sustainable alternative BET assay for the future

The PyroGene™ rFC is a recombinant form of horseshoe crab Factor C. It only contains Factor C and none of the other factors found in the traditional LAL cascade, so it retains all the endotoxin reactivity of LAL, but with greater lot-to-lot consistency than traditional LAL.

Standard curves generated from different lots of the PyroGene™ rFC Assay show remarkable consistency providing more reproducible results and less variable recovery values for PPCs (Positive Product Controls).



Lot-to-lot consistency

Figure 2: Endotoxin standard curves using six different lots of rFC. Lot-to-lot standard curves exhibit excellent reproducibility.

Assay validation according to USP <1225> and ICH Q2B

The PyroGene™ rFC Assay is equivalent to other photometric endotoxin detection methods that use LAL to detect endotoxin according to the parameters listed in United States Pharmacopeia (USP) <85>. Currently, the recombinant Factor C method is considered an alternative to LAL-based methods and requires validation according to USP <1225> "Validation of Compendial Procedures." Parameters evaluated in USP <1225> include specificity, linearity, precision, accuracy, and limit of detection.

A global, multi-center study demonstrated that the recovery of endotoxin from water and other tested products using the PyroGene™ rFC Assay was comparable to that of LAL-based methods. Lonza validated 10 representative products according to USP <1225>. The products tested in the multi-center study included items such as Water for Injection, Lactated Ringer's Injection USP, 0.9% Sodium Chloride Injection USP, Erythropoietin, Albumin (Human) USP 25% Solution, Vancomycin HCl USP, and Hemodialysate. Each product was spiked with 0.1 EU/mL of endotoxin and tested with the kinetic chromogenic and rFC methods by three analysts at six different locations. The rFC results were comparable or better than conventional LAL methods.

The complete results of the assay validation were published in the Pharmacopeial Forum Vol. 36(1) [Jan.–Feb. 2010]. The following are some of the results of our comparability studies.

PyroGene™ rFC Assay delivers a quantitative range of 0.005– 5.0 EU/mL in a single step

Linearity

Linearity of the standard curve is assessed with the correlation coefficient. The absolute value of the correlation coefficient, r, must be greater than or equal to 0.980 for the range of endotoxin concentrations tested.

The results shown below are of six standard curves run on the PyroGene™ rFC Assay by three different analysts at six separate sites. The PyroGene™ rFC Assay produced an average %CV of 0.996, which far exceeds the minimum requirements of USP <1225>.

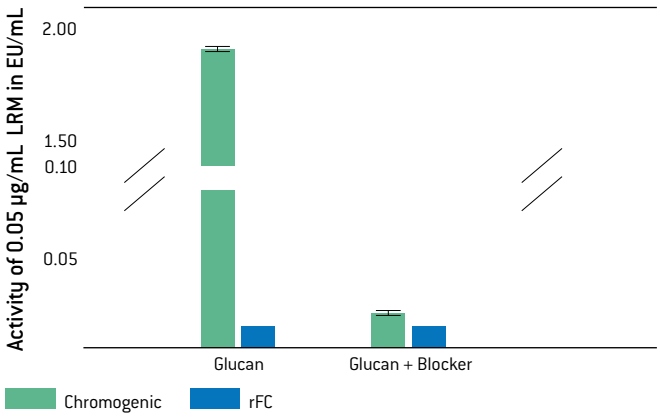
Standard curve characteristic	Mean	CV%	Range	
			Min	Max
Correlation(r)	0.996	0.1%	0.995	0.998
Slope	0.865	1.9%	0.831	0.901
Y-intercept	3.398	1.1%	3.307	3.450

Table 2: Linear regression statistic summary, based on 18 standard curves, by three analysts at six sites.

Specificity

The PyroGene™ rFC Assay makes use of only Factor C which is specifically activated by endotoxins. This makes the PyroGene™ rFC Assay more predictable with less inhibition or enhancement.

Unlike LAL, the PyroGene™ rFC Assay does not contain Factor G, which is reactive to glucans. In LAL assays the reactivity of Factor G can be variable depending on the manufacturer and their lysate formulation. Since the PyroGene™ rFC Assay cannot react with glucans, no false-positive results are obtained due to glucan interaction.



Accuracy

Accuracy is the closeness of test results obtained by that procedure to the true value. "Closeness" can be assessed by determining whether assay results fell within 2-fold of the known sample concentration (between 50 to 200%).

Results from this study show that the PyroGene™ rFC Assay more accurately detected the 0.0316 EU/mL spike when compared to the kinetic chromogenic method. 81.5% of the results were within 25% of the known concentration of the spike versus only 74.1% of the results using the kinetic chromogenic method.

Accuracy was also analyzed by comparing the recovery of a 0.1 EU/mL spike in water for three different test methods. The endotoxin content found was 0.092 EU/mL with the chromogenic method; 0.112 EU/mL with the turbidimetric assay; and 0.105 EU/mL with the PyroGene™ rFC Assay, (Table 4).

		rFC	Chromogenic
0.0316 EU/mL sample	Mean standard deviation	0.0311 EU/mL 0.0063 EU/mL	0.0366 EU/mL 0.0070 EU/mL
	% of results within ±25% of known concentration[b]	81.5%	74.1%
0.316 EU/mL sample	Mean standard deviation	0.392 EU/mL 0.056 EU/mL	0.421 EU/mL 0.065 EU/mL
	% of results within ±25% of known concentration[b]	44.4%	29.6%
3.16 EU/mL sample	Mean standard deviation	3.54 EU/mL 0.36 EU/mL	3.22 EU/mL 0.50 EU/mL
	% of results within ±25% of known concentration[b]	92.6%	87.0%

Table 3: Accuracy of the PyroGene™ rFC Assay using three different endotoxin concentrations.

	Chromogenic	Turbidimetric	rFC
Average EU/mL	0.092	0.112	0.105
Std. Dev.	0.009	0.009	0.012

Table 4: Recovery of a 0.1 EU/mL spike in Water for Injection as tested by multiple methods.

Figure 3: Comparison of glucan activity between kinetic chromogenic LAL and rFC. The false positive signal from the LAL assay is reduced in the presence of a glucan blocker. rFC does not detect any glucan activity since the assay is endotoxin specific.

Precision

Precision as defined by USP <1225> is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample.

The precision of an analytical method is usually expressed as the standard deviation and coefficient of variation (%CV) of multiple measurements.

Table 5 shows recombinant Factor C tests conducted at 3 locations. Each location was provided with 3 samples of different endotoxin concentrations. The low sample was 0.03 EU/mL, the medium sample was 0.3 EU/mL and the high sample was 3 EU/mL. Eighteen tests were performed per location, for a total of 54 tests.

The coefficient of variation was calculated for the individual locations as well as the overall CV. The table below compares each sample with both the rFC and kinetic chromogenic method. CVs for the PyroGene™ rFC Assay were comparable to the CVs for the chromogenic assay, with the PyroGene™ rFC Assay results of 16% for the low sample, 17% for the medium sample and 11% for the high sample; this compared to 19%, 20% and 16% for the chromogenic assay, respectively.

Sample EU/mL	rFC %CV	Chromogenic %CV
0.0316	16	19
0.316	17	20
3.16	11	16

Table 5: Observed %CV from all sites, N = 54 (3 locations x 18 tests per location) %CV pooled within location.

2012 June

FDA issues a revision of the "Guidance for Industry Pyrogen and Endotoxins Testing: Questions and Answers" document which allows for use of a recombinant Factor C based assay as an alternative to LAL-based assays.*

2015 July

Recombinant Factor C is officially recognized by the European Pharmacopoeia (Ph. Eur.) as an alternative endotoxin detection methodology to the LAL and Rabbit Pyrogen Tests in the new draft of Chapter 5.1.10.*

2016 July

European Pharmacopeia Chapter 5.1.10 officially is effective.

2018 September

The U.S. Food and Drug Administration approves the first drug using a recombinant method for endotoxin testing instead of traditional LAL-based methods for a monoclonal antibody drug treatment for the prevention of migraines in adults.

2018 December

The European Pharmacopeia releases a draft of their new compendial Chapter 2.6.32 dedicated to the recombinant Factor C method.

2019 January

Recombinant Factor C (rFC) is listed and described as a new compendia method for bacterial endotoxin testing in the Chinese Pharmacopeia, following the EP, JP, and FDA. The 4th version of the Chinese Pharmacopeia will be effective in 2020.

*The rFC assay will still be considered an "Alternative Test", subject to the validation requirements of USP <1225> or ICH Q2B. Regulatory authorities will accept the test results of the recombinant Factor C assay, but a validation study must be performed for each product that will be tested using this method. (Validation studies are used to compare the alternative and compendial method, and verify the equivalence between the two methods of the assay. Post-validation, it is necessary to follow up with the appropriate regulatory filing for the drug product or device.)

Equivalency to LAL methods

The PyroGene™ rFC Assay has the ability to detect endotoxin in comparable fashion to LAL-based methods. USP 28-NF 33 General Notices states that alternative testing methods, such as rrecombinant Factor C can be used if the alternative method provides advantages in accuracy, sensitivity, precision, selectivity, or adaptation to automation. Such alternative methods need to be validated as described in the general chapter, Validation of Compendial Procedures <1225>.

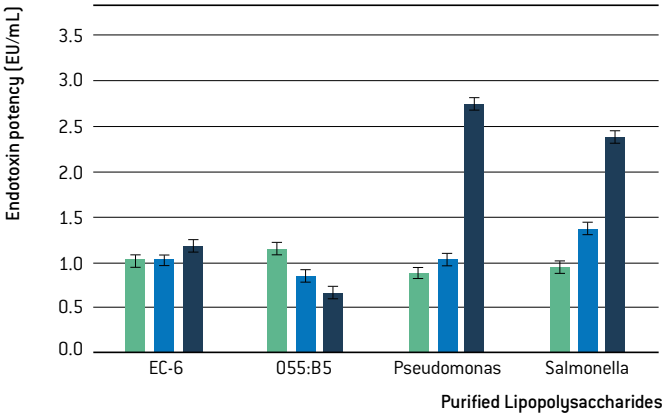


Figure 4: The rFC method recognizes endotoxin from different source materials similar to other photometric procedures, demonstrating comparability to the LAL-based methods.

Regulatory status of the PyroGene™ rFC Assay

510(k) submissions have been cleared by the FDA using the PyroGene™ rFC Assay as a final release test. Lonza has also prepared and submitted a comprehensive FDA Master File.

Accepted by several global regulatory authorities, including the FDA and the European Pharmacopeia, the recombinant Factor C method is a recognized, comparable endotoxin detection method to LAL-based assays.

Product validation of PyroGene™ rFC Assay

2 days	0.5 days	>0.5 days (min.)	>1.5 days (min.)	0.5 days
Preparation	**Initial qualification**	**Inhibition/ enhancement testing**	**Validation of alternative method**	**Product validation**
• IOP/Q • Sensitivity assay for rFC • Initial qualification of current and rFC method and analyst	• Not necessary if previously qualified reagent lots are used	• Only for rFC • Time depending on extent of testing required to find a suitable dilution/pre-treatment	• Tests with both current and rFC method • Good planning can save time	• 3 production lots

Table 6: Validation timeline at-a-glance

Reading the general chapter Validation of Compendial Procedures <1225> in the USP 28-NF 33 General Notices can seem intimidating, and appear to be a lot of work to validate the PyroGene™ rFC method for your product. However, the actual time could be as little as 5 days, (Table 6).

PyroGene™ rFC product validation process and timeline
A possible validation scheme is outlined below (one validation can be accomplished in as little as 5 days assuming that the product has been previously validated with a quantitative LAL method). Table 6 shows the validation scheme is identical to that which would be needed for any LAL-based method with just the addition of one extra step, "Validation of Alternative Method".

1. Preparing to start the validation
   - Comparison between a current kinetic photometric method and the rFC method
   - Experiments before starting validation
   - Equipment: IOPQ
   - Analysts: initial qualification of both current and rFC method
     - Sensitivity assay for PyroGene™ rFCAssay lot used in the following experiments (if different from OQ)
   - Current method: product treatment as validated

2. Initial qualification
   - Current method (kinetic chromogenic or turbidimetric LAL)
   - PyroGene™ rFC Assay
     - Approval criteria: $|R| \geq 0.980$
     - Not necessary if performed with the same lot numbers before (e.g. during IOPQ)

3. Inhibition/enhancement
   - Current method: not necessary, as product has already been validated
   - PyroGene™ rFC Assay
     - Use pre-treatment and dilution for current method as lead
     - Dilution series including PPC
     - Approval criteria:
       - $|R| \geq 0.980$
       - PPC recovery between 50-200%
         - Select dilution for further validation

4. Validation of alternative method
   - Accuracy and Precision
     - Both current and rFC method
       - 3 product samples
       - 3 independent assays on each sample (same sample with fresh lysate and standard curve)
       - Pre-treatment and dilution as determined in validation (current method) or Inhibition/Enhancement test (PyroGene™ rFC Assay)
       - Pre-treated/diluted sample spiked with 3 endotoxin concentrations (low, middle and high; e.g. 0.03 EU/mL, 0.3 EU/mL, 3 EU/mL)
       - In triplicate
     - Accuracy: calculate for each method and compare
       - Mean and standard deviation for low/middle/high spike
       - % of results within 50-200% of known concentration
     - Precision: calculate for each method and compare
       - Mean, standard deviation and CV% for low/middle/high spike
   - Specificity: included in accuracy and precision
   - Range and Limit of Quantitation
     - Not necessary for current method
     - PyroGene™ rFC Assay
       - Data of "Accuracy and Precision" tests might be used (if spike was ≤ ERL, taking dilution factor into account)
       - If not: same tests as "Accuracy and Precision" with different spikes and freshly made product spikes for each test
       - Calculate
         - Mean EU/mL and % recovery of known value
         - Coefficient of correlation
       - Acceptance criteria
         - 50-200% recovery
         - $|R| \geq 0.980$
   - Linearity: uses correlation coefficients of previous assays for calculation

5. Product validation
   - 3 production lots
   - Approval criteria:
     - $|R| \geq 0.980$
     - PPC recovery between 50-200%

*We offer a full Validation Protocol that be followed for your convenience. For further information and to submit, please visit www.lonza.com/pyrogene.

The PyroWave™ XM Fluorescence Reader is a part of the quantitative endotoxin detection system that supports the PyroGene™ rFC Endpoint Fluorescent Assay. This reader replaces the Lonza FLx800™ LBS reader, bringing in a new generation in fluorescence technology to users of the PyroGene™ rFC Assay. Optimized specifically for Lonza's PyroGene™ rFC Assay, this reader brings new and improved technology to the laboratory. The PyroWave™ XM reader offers numerous enhancements in incubation, optics, automation capability, and overall reader maintenance and robustness. Along with WinKQCL™ Endotoxin and Analysis Software (version 5.3 and higher), Lonza delivers a high performance and easy-to-use system for users interested in an alternative to horseshoe crab-based endotoxin detection test methods.

Contact your local Lonza sales representative for more information about on-site service and preventative maintenance contracts designed to help ensure that your instrument is working properly.

Read capabilities	Fluorescence, luminescence**, TRF**, FP**
Read position	Top read
Light source	Xenon flash lamp
Detection	High performance photo multiplier tube
Fluorescence sensitivity	Fluorescein @ 1 pM/well in a 96-well plate
Wavelength range	200 to 850 nm**
Filters	One easy-to-swap filter cube with the following filter configuration: **Additional filter cubes and filters must be purchased from the reader manufacturer for additional wavelengths and read capabilities
Temperature control	±0.2 °C at 37 °C
Power	100 – 240 Volts AC 50/60 Hz
Dimensions	39.1 cm W x 47.2 cm D x 32.8 cm H (15.4" W x 18.6" D x 12.9" H)
Weight	22.5 kg (50 lbs)

Table 7: PyroWave™ XM Fluorescence Reader Specifications

Benefits

- Improved PyroGene™ rFC Assay performance
- Reduced reader maintenance
- Automation compatible
- High performance optics
- 4-Zone™ Incubation at 37°C

Applications

- Parenterals
- Biologics
- Medical devices
- Dialysis
- Compounding
- Water analysis
- R&D

Assay type	Excitation filter (nm)	Emission filter (nm)	Dichroic filter (nm)
PyroGene™	380/20	440/30	400
Fluorscein	485/20	528/20	510

Additional filter cubes and filters must be purchased for additional wavelengths and read capabilities.

Table 8: Assays performed on the PyroWave™ XM Reader.

Summary

Lonza's endotoxin detection products have been available since the 1970s. For four decades, Lonza has been leading the way in innovation for endotoxin detection products, methods and software.

The PyroGene™ rFC Assay is the evolution of the LAL test. Combining recombinant technology with the horseshoe crab's endotoxin sensitive protein, Lonza has developed an equivalent, reliable and sustainable endotoxin detection method for the future.

Ordering information

Product name	Sensitivity EU/mL	No. of tests	Contents	Catalog No.
PyroGene™ Recombinant Factor C Assay	0.005 to 5	192 test kit	2 x 96 tests/vial rFC enzyme solution 2 x 6 mL vial fluorogenic substrate 2 x 5 mL vial rFC assay buffer 2 x 10 ng/vial endotoxin 2 x 30 mL vial LAL Reagent Water	**50-658U**
PyroGene™ Recombinant Factor C Assay	0.005 to 5	2880 test kit	30 x 96 tests/vial rFC enzyme solution 30 x 6 mL vial fluorogenic substrate 30 x 5 mL vial rFC assay buffer 10 x 10 ng/vial endotoxin	**50-658NV**
PyroWave™ XM Fluorescence Reader			Fluorescence reader	**25-345S**
WinKQCL™ 6 Software Package			Endotoxin Detection & Analysis Software	**25-611**

* Custom product which requires 6-week lead time for ordering.

Lonza Walkersville, Inc. – Walkersville, MD 21793

For *In Vitro* Use Only. Not intended to detect endotoxemia in man or animals, or for use in clinical diagnosis, patient management, or for the qualification of blood or blood products.

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Quality Management Systems and Enviromental certified according to EN ISO 9001, ISO 13485 and EN ISO 14001

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