“Error-proofing” and “future-proofing”: updating the bacterial endotoxins test with automation and recombinant reagents

Lonza

Pharma & Biotech

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Objectives

Use of a recombinant Factor C reagent for endotoxin detection that shows equivalent performance to the Limulus Ambocyte Lysate (LAL) bacterial endotoxin test (BET) helps meet sustainability goals and comply with principles of the 3Rs (replacement, reduction, refinement of animal use). Application of a robotic platform allows more accurate and precise assay performance, maximizing efficiencies in workstreams. Combining both of these components error-proofs and future-proofs assay execution in your laboratory. This presentation will present data and discuss both of these concepts to demonstrate laboratory process optimization.

Abstract

The use of robotics and automation reduces error levels common to many manual processes, thus saving time and resources spent on such tasks. Studies comparing manual and automated pipetting and mixing in the BET test show improved precision and accuracy with the automated method. With such automation, laboratory analysts are freed to perform other tasks while a robot performs the manual tasks, resulting in more efficient use of that analyst’s time. Automation of BET methods minimizes the level of interaction with lab analysts, resulting in a reduced level of repetitive motion injuries in line with promulgated regulations for workers protection. In addition to the use of robotics for error-proofing the assay, there is also a need to future-proof the supply chain of reagents. Using a recombinant reagent allows removal of the live animal source thus complying with the “3 R” principles, multiple facility production, and results in a more stable supply chain of a reagent with less lot-to-lot variability. Recombinant Factor C (rFC) based endotoxin detection assays demonstrate equivalent performance when compared to classical LAL-based assays. This presentation will demonstrate that combining the concepts of automation and recombinant reagents results in an error-proofed and future-proofed BET assay process that optimizes laboratory operations.

Recombinant Factor C (rFC) based endotoxin detection assays demonstrate equivalent performance when compared to classical LAL-based assays.

The majority of parenteral drugs and implantable medical devices are tested for Gram-negative bacterial endotoxin using reagents prepared from the circulating amebocytes found in the blood of the American horseshoe crab, *Limulus polyphemus*. Variations of this method are described in the USP General Chapter <85> Bacterial Endotoxins Test[1]. This chapter describes the gel clot LAL method and the various kinetic and endpoint photometric methods. While the gel clot method is still reserved as the official referee test in cases of dispute, it is implied, by their inclusion, that the photometric methods are acceptable for routine use, if appropriately validated. rFC methods start with the same Factor C as the compendial assays, but do not need the signal amplification steps (Figure 1). The assay also yields comparable results as the compendial assay (Figure 2). rFC methods offer several distinct advantages over LAL-based methods[2], specifically:

- Eliminates the need to collect and bleed horseshoe crabs, a species facing increased harvesting restrictions
- Provides a non-animal-based method that is needed due to the growing use of LAL for endotoxin testing into expanding markets (example: Cell Therapy)
- Protects the supply of endotoxin detection reagents to the pharmaceutical and medical device industries, should a natural or man-made disaster impair the ability of one of the three major LAL manufacturers to harvest horseshoe crabs
- Does not pose a threat to species that rely on the horseshoe crab for survival (i.e. the Red Knot, *Calidris canutus*)
- Reduces the lot-to-lot variability inherent in animal-derived products like LAL
- Offers more specific endotoxin detection than LAL because it does not contain other horseshoe crab blood components (i.e. Factor G), which can react with non-endotoxin substances (LAL Reactive Materials (LRM)/glucans) and cause false-positive reactions[3,4]. The release of entire lots of parenteral drugs or medical devices may be delayed while the false-positive results are evaluated; in some cases material may be unnecessarily destroyed, raising implications for shortages)
- Allows for the year-round production of the active ingredient (rFC), rather than the seasonal harvesting of horseshoe crab blood during the warmer months
- Does not require toxic or otherwise carcinogenic chemicals to manufacture
- Does not require lyophilization of the active ingredient, resulting in increased manufacturing efficiencies and ease-of-use to end-users. As the active ingredient can be stored in liquid form, this facilitates its use with an on-line endotoxin detection system for water purification systems, which is in line with the FDA Process Analytical Technology initiative

Figure 1 below illustrates that rFC shares the same initial enzyme as traditional LAL assays, while Figure 2 illustrates the comparability of endotoxin recovery between rFC and classical LAL assays.

Figure 1

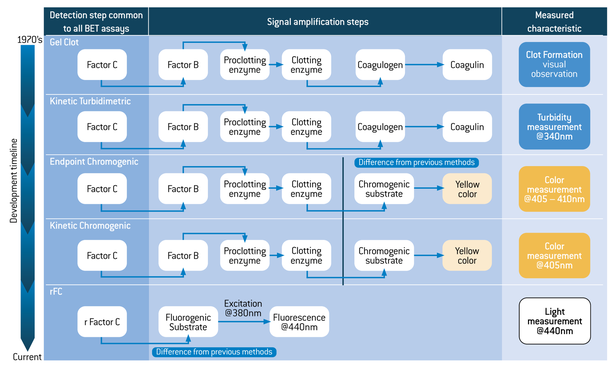
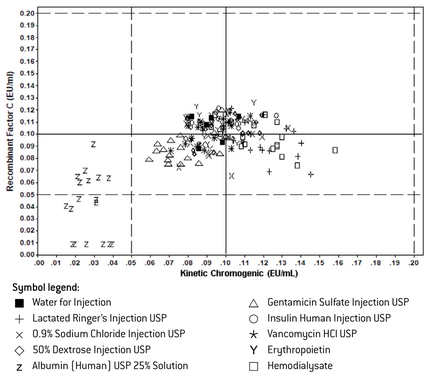


Figure 2



Using robotics and automation reduces error levels common to many manual processes, thus saving time and resources spent on retests. Studies comparing manual and automated pipetting and mixing in the BET test show improved precision and accuracy with the automated method

Our laboratories performed studies to demonstrate that endotoxin standard preparation by automated pipette mixing on the PyroTec™ PRO System is equivalent to manual preparation of standards using vortex mixing.

Lonza’s Kinetic-QCL™ Kinetic Chromogenic LAL Assay Kit provided the necessary endotoxin standard and LAL reagents needed to complete this testing. The CSE standard was reconstituted with the required amount of LAL Reagent Water (LRW) indicated on the Certificate of Analysis for the KQCL kit. The CSE vial was vigorously mixed for 15 minutes on a vortex mixer, and a set of standards prepared by dilution with LRW by both the PyroTec™ PRO System and manually by a skilled analyst. The PyroTec™ PRO System diluted and mixed by the repeated aspirating and dispensing of 700 µL from a 1 mL total volume seven times for each concentration. Manual dilutions were mixed by vortexing for one minute, as described in the current Kinetic-QCL™ Kinetic Chromogenic LAL Assay Kit Insert.

The goal in mixing dilutions was to obtain homogeneous solutions at each concentration. Pipette mixing was successful if the standards produced exhibited similar characteristics to those of the manually mixed standards using a vortex mixer. Each standard curve derived by either manual mixing with a vortex mixer, or pipette mixing on the robot had to meet the system suitability criteria to be included in the final analysis.

The system suitability criteria that must be met in all Lonza kinetic chromogenic assays were the following:

- Standard Curve with Correlation Coefficient between -1.000 to -0.980, Slope -0.400 to -0.100, and Y-Intercept between 2.500 to 3.500
- Positive Product Control (PPC) with % Recovery between 50% to 200%, and Endotoxin Prediction of a 0.5 EU/mL PPC between 0.25 EU/mL to 1.0 EU/mL
- Endotoxin Standards with % CV of < 10 %
- Run Temperature maintained between 36 – 38 °C

This study was comprised of twelve separate comparison tests of CSE standard dilutions with concentrations from 50 EU/mL to 0.005 EU/mL and Blank controls, LRW samples, and LRW samples plus PPC.

The PyroTec™ PRO System followed commands from a WinKQCL™ Software template to instruct the analyst where to place dilution tubes and the pre-prepared 50 EU/mL CSE on the deck. While the instrument made the automated dilutions for the standard curve, the analyst manually diluted and vortex mixed the standards.

The analyst manually loaded standards from each method, blanks, and test samples to the assay plate. The PyroTec™ PRO System produced standard was loaded into the first two columns with Blank and test sample. The analyst loaded the PPC spike from this standard into the sample PPC wells. The manually produced standard, blank, sample, and sample plus PPC spike were loaded into the third and fourth columns.

Separate templates for the automation standard with sample and the manual standard with sample were run as a Merged Plate template in each assay run.

Assay reports, generated automatically at the completion of each assay run, were evaluated and the data saved for analysis at the completion of all twelve tests.

Results

All system suitability criteria were met in the analyzed runs. Run temperatures were verified to be within 36 – 38°C for all runs. In all runs the lowest standard reaction time was less than the blank reaction time.

The initial data analysis considered the method overlay of all 12 standard curves from each of the two preparation methods. These graphs show little to no variability from run to run.

See Figures 3 and 4 below.

Figure 3

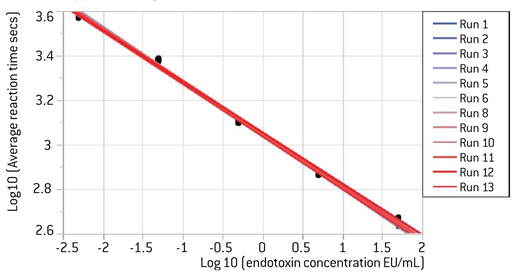
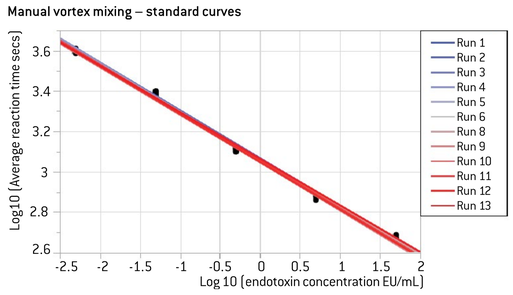


Figure 4



Product Testing using the Kinetic-QCL™ Kinetic Chromogenic LAL Assay on PyroTec™ PRO System

This study evaluated the Automated Endotoxin Assay as compared to manual endotoxin testing. The goal was to demonstrate that the automation assay produces equivalent data to an analyst’s manual data. Assays performed on both manual and automation platforms included a panel of 10 samples (Figure 6, below). Some samples required preparation prior to testing including pH correction or dilution. The samples included traditionally challenging samples for the kinetic assay due to enhancement or inhibition, samples with known spikes of endotoxin, and were analyzed using Kinetic-QCL™ Kinetic Chromogenic LAL Assay Kits.

This initial study included each of the 10 samples tested at Maximum Valid Dilution (MVD), 1/2 MVD, and 1/10 MVD. The samples were tested as neat (unspiked) and spiked with endotoxin at 5.0 EU/mL. The analysts tested the entire panel of products including spikes two separate times and on both platforms.

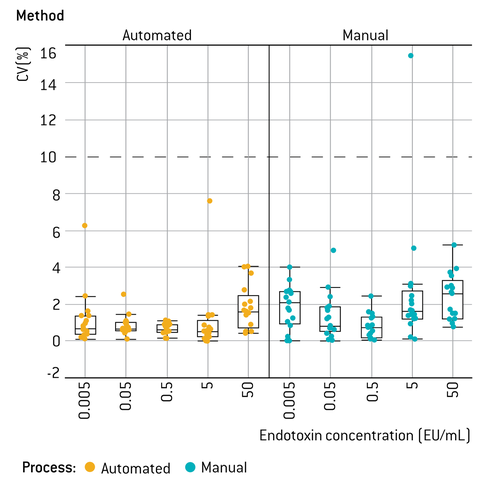
Results

Figure 5 shows that results from the manual assay were comparable to those of the automated assay for all products tested. Figure 6 shows that the Coefficient of Variation (CV) within dilutions of the CSE used for the standard curve are tighter than the CVs for the manual process, demonstrating less variability of results in automated assays than manual assays.

Figure 5

Sample + PPC	N		Mean reaction time		Std dev over assays		0.1Pooled % CV among repl wells	
	Manual	Auto	Manual	Auto	Manual	Auto	Manual	Auto
10 mM MgCl2 Solution	4	4	1,080	1,001	19	14	1.7	1.5
Albumin USP 25% Solution	2	2	1,388	1,189	20	9	1.4	0.8
50% Dextrose injection USP	3	3	1,092	1,162	18	10	1.6	0.8
Dulbecco’s Modified Eagle’s Medium + Ham’s F12	3	3	969	1,018	6	17	0.6	1.7
Gentamicin Sulfate injection USP	3	3	1,233	1,008	5	2	0.5	0.2
Humulin R	3	3	1,066	965	18	4	1.7	0.4
0.5% L-Glutamine Acid – 1	3	3	1,084	1,050	5	7	0.5	0.7
0.5% L-Glutamine Acid – 2	3	3	1,086	1,051	9	2	0.8	0.2
1.0% L-Leucine – 1	3	2	1,046	1,045	7	5	0.6	0.5
1.0% L-Leucine – 2	3	2	1,108	1,101	26	6	2.2	0.5
1.0% L-Lysine Monohydrochloride – 1	3	3	1,164	1,128	6	11	0.5	1.0
1.0% L-Lysine Monohydrochloride – 2	3	3	1,174	1,145	4	5	0.3	0.4
1.0% L-Phenylaianine	3	3	1,145	1,129	5	6	0.4	0.5
1.0% L-Proline (00119NC)	3	1	1,091	1,004	9	12	0.8	1.2
1.0% L-Proline (00119-1)	3	3	1,112	995	23	4	2.0	0.4
1.0% L-Proline (00119-2)	3	3	1,065	987	10	4	0.9	0.4
1.0% L-Threonine – 1	3	3	1,119	1,063	8	9	0.7	0.9
1.0% L-Threonine – 2	3	3	1,107	1,048	2	5	0.1	0.5
Procrit	3	2	1,108	977	8	5	0.8	0.5

Figure 6



Conclusion

Overall, the standard and sample results were very similar between Automation and Manual methods of performing the Kinetic-QCL™ Kinetic Chromogenic LAL Assays.
All samples had at least one passing MVD dilution scheme in each method except for the Albumin samples. Neither method was able to obtain passing results in the original dilutions.
Lonza recommends treating samples like Albumin with Pyrosperse™ Dispersing Agent before performing the kinetic assays.
The PPC % recovery in the samples was closer to 100 % while in the Manual method they are closer to 125 %. The PPC % CV were low for both methods with the Automation method producing tighter results overall.
All of the standards met the specifications for passing in the Kinetic-QCL™ Kinetic Chromogenic LAL. The standards were very similar between the methods. The Automation standard’s Y-intercept values were slightly broader, but the % CV values among replicates were tight.

Putting it all together: Error-proofing and Future-proofing

The laboratory performed a reliability study using Recombinant Factor C (rFC) on the PyroTec™ PRO System. Once programmed, the robot was able to repeatedly and accurately perform dilution series by pipetting Control Standard Endotoxin (CSE) in a series of 1:10 dilutions, then plate those dilutions into the proper wells, pipette PyroGene™ Recombinant Factor C reagent into each well, and then move the prepared plate into the fluorescence reader. This saved approximately one hour of analyst time over manual preparation in each assay setup. Once those steps were completed, the WinKQCL™ Software took over to monitor the readers’ temperature, timing, wavelength, and generated fluorescence to calculate the amount of endotoxin in each well. As shown, the standard curves in Figure 7 lay nearly on top of each other, and are practically parallel. The histograms in Figures 8 and 9 illustrate the precision and accuracy that may be achieved using a combination of the animal-friendly and renewable PyroGene™ and the PyroTec ™ PRO System.

Figure 7

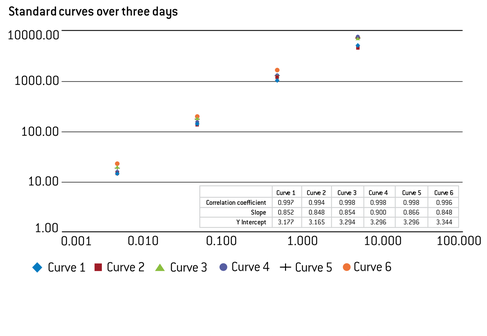


Figure 8

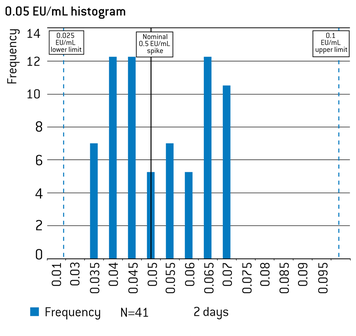
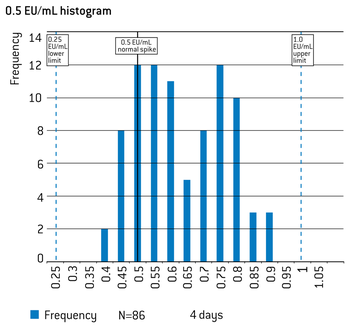


Figure 9



As demonstrated in this presentation, removing the analyst from such labor-intensive tasks and relying on robotics reduces the chance for human error in the process, and produces less assay variation thus “error-proofing” the assay. Relying on recombinant sources of materials used in endotoxin testing provides a renewable endotoxin detection assay guarding the safety of parenteral products without relying on animals as a source of raw materials, thus “future-proofing” the availability of the reagents. These combined attributes make the use of the PyroGene™ rFC assay with the PyroTec™ PRO System ideal for high-throughput laboratories.

References

[1] United States Phamacopeia, 30th rev. U.S. Pharmacopeial Convention, Inc., Rockville, MD; Bacterial Endotoxins Test. General Chapter <85>.
[2] Loverock B, Simon B, Burgenson A, Baines A. A Recombinant Factor C Procedure for the Detection of Gram-Negative Bacterial Endotoxin. Pharmacopeial Forum. Vol 36(1) Jan-Feb 2010.321-329.
[3] Morita T, Tanaka S, Nakamura T, Iwanaga S. A new (1→3)-β-D-glucan-mediated coagulation pathway found in Limulus amebocytes. FEBS Lett. 1981; 129: 318-321.
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