Application Note

Automated set-up of the Lonza PyroGene® Recombinant Factor C (rFC) Assay for endotoxin detection on the ASSIST PLUS

Introduction

The Lonza PyroGene rFC Assay is an alternative to the traditional limulus amebocyte lysate (LAL) assay, which is widely used to screen for bacterial endotoxin contamination in human and animal parenteral pharmaceuticals and medical devices. The rFC test is used in both high and low throughput laboratories and, unlike the LAL assay, is not derived from horseshoe crab blood.
Setting up the test requires preparation of 10-fold diluted standards from the endotoxin stock solution supplied. Standards and samples are tested in duplicate in a 96 well plate. To check for product inhibition, positive product controls (PPCs) – samples spiked with a known concentration of endotoxin – are tested alongside the samples. Following the initial plating of the standards, samples and PPCs, a 10-minute pre-incubation is performed, during which the user prepares a working solution consisting of the fluorogenic substrate, assay buffer and rFC enzyme.

This application note demonstrates that the preparation of the standards, samples, PPCs and blanks – and their addition to the 96 well plate – can be easily automated on the ASSIST PLUS pipetting robot using a D-ONE single channel pipetting module. Addition of the working reagent is then completed using a VOYAGER adjustable tip spacing multichannel pipette. Automation of all the pipetting steps reduces the opportunity for pipetting errors, and ensures assay robustness and reproducibility. The key quality indicators in this assay are the correlation coefficient of the standard curve and coefficient of sample variation (CV).

Key benefits:

- Automated preparation of standard dilutions eliminates pipetting errors that could invalidate entire runs.
- Users can perform testing of full or partial plates by using the D-ONE single channel pipetting module in combination with the ASSIST PLUS.
- Error-free pipetting ensures replicate samples with tight CV values, reducing the likelihood of repeat testing.
- On-screen prompts guide the user through instrument set-up.

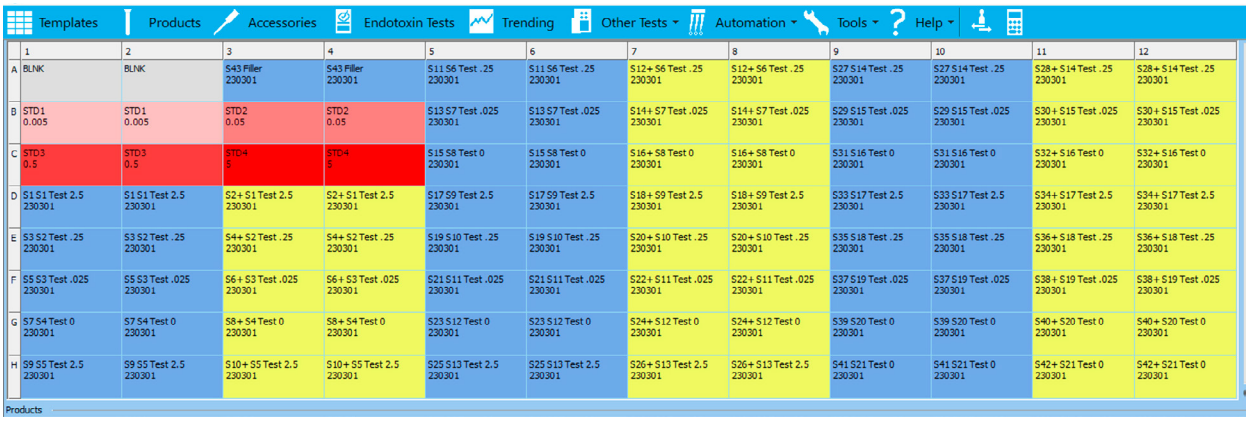
Overview: How to perform the PyroGene rFC Assay

ASSIST PLUS

Application Note

INTEGRA

The PyroGene rFC Assay is a fluorogenic assay that requires a fluorescence microplate reader – such as the PyroWave® XM Fluorescence Reader, paired with the WinKQCL® Endotoxin Detection & Analysis Software – to measure endotoxin values. Prior to setting up the plate, a template is prepared in WinKQCL software. **Figure 1** shows the WinKQCL software template that was developed for use with the ASSIST PLUS. This template allows up to 21 samples to be tested in duplicate on one plate, with paired PPCs. It is designed to provide optimal flexibility for varying numbers of samples, while still allowing use of an 8 channel pipette to deliver the rFC working solution to the plate, as described in the assay instructions for use.



	1	2	3	4	5	6	7	8	9	10	11	12
A	BLNK	BLNK	S43 Filler 230301	S43 Filler 230301	S11 S6 Test .25 230301	S11 S6 Test .25 230301	S12+ S6 Test .25 230301	S12+ S6 Test .25 230301	S27 S14 Test .25 230301	S27 S14 Test .25 230301	S28+ S14 Test .25 230301	S28+ S14 Test .25 230301
B	STD1 0.005	STD1 0.005	STD2 0.05	STD2 0.05	S13 S7 Test .025 230301	S13 S7 Test .025 230301	S14+ S7 Test .025 230301	S14+ S7 Test .025 230301	S29 S15 Test .025 230301	S29 S15 Test .025 230301	S30+ S15 Test .025 230301	S30+ S15 Test .025 230301
C	STD3 0.5	STD3 0.5	STD4 5	STD4 5	S15 S8 Test 0 230301	S15 S8 Test 0 230301	S16+ S8 Test 0 230301	S16+ S8 Test 0 230301	S31 S16 Test 0 230301	S31 S16 Test 0 230301	S32+ S16 Test 0 230301	S32+ S16 Test 0 230301
D	S1 S1 Test 2.5 230301	S1 S1 Test 2.5 230301	S2+ S1 Test 2.5 230301	S2+ S1 Test 2.5 230301	S17 S9 Test 2.5 230301	S17 S9 Test 2.5 230301	S18+ S9 Test 2.5 230301	S18+ S9 Test 2.5 230301	S33 S17 Test 2.5 230301	S33 S17 Test 2.5 230301	S34+ S17 Test 2.5 230301	S34+ S17 Test 2.5 230301
E	S3 S2 Test .25 230301	S3 S2 Test .25 230301	S4+ S2 Test .25 230301	S4+ S2 Test .25 230301	S19 S10 Test .25 230301	S19 S10 Test .25 230301	S20+ S10 Test .25 230301	S20+ S10 Test .25 230301	S35 S18 Test .25 230301	S35 S18 Test .25 230301	S36+ S18 Test .25 230301	S36+ S18 Test .25 230301
F	S5 S3 Test .025 230301	S5 S3 Test .025 230301	S6+ S3 Test .025 230301	S6+ S3 Test .025 230301	S21 S11 Test .025 230301	S21 S11 Test .025 230301	S22+ S11 Test .025 230301	S22+ S11 Test .025 230301	S37 S19 Test .025 230301	S37 S19 Test .025 230301	S38+ S19 Test .025 230301	S38+ S19 Test .025 230301
G	S7 S4 Test 0 230301	S7 S4 Test 0 230301	S8+ S4 Test 0 230301	S8+ S4 Test 0 230301	S23 S12 Test 0 230301	S23 S12 Test 0 230301	S24+ S12 Test 0 230301	S24+ S12 Test 0 230301	S39 S20 Test 0 230301	S39 S20 Test 0 230301	S40+ S20 Test 0 230301	S40+ S20 Test 0 230301
H	S9 S5 Test 2.5 230301	S9 S5 Test 2.5 230301	S10+ S5 Test 2.5 230301	S10+ S5 Test 2.5 230301	S25 S13 Test 2.5 230301	S25 S13 Test 2.5 230301	S26+ S13 Test 2.5 230301	S26+ S13 Test 2.5 230301	S41 S21 Test 0 230301	S41 S21 Test 0 230301	S42+ S21 Test 0 230301	S42+ S21 Test 0 230301

Figure 1: Lonza rFC template in WinKQCL software. Red wells: standards; blue wells: samples in duplicate (wells A3 and A4 are empty); yellow wells: PPCs tested in duplicate.

A 5-300 µl D-ONE single channel pipetting module is first used to prepare standard dilutions. Following preparation of the standards, PPC is added to the designated wells using repeat dispense mode. Next, standards and samples are added to the plate in duplicate according to the above template, with samples added to both clean and PPC-spiked wells. Once all samples are added, the plate is pre-incubated at 37 °C for 10 minutes. During the incubation, the user prepares the working reagent in an INTEGRA 10 ml SureFlo™ reagent reservoir, which is added to the plate at the end of the pre-incubation period using a 300 µl VOYAGER 8 channel pipette on the ASSIST PLUS.

TIPS:

- Use pyrogen-free certified GRIPTIPS® pipette tips in combination with SureFlo reservoirs to ensure accurate results. 10 ml SureFlo reservoirs require a dead volume of less than 30 µl.
- 300 µl long GRIPTIPS can access sample volumes of below 1 ml in 13x100 mm Lonza pyrogen-free test tubes, and will never leak or fall off.
- PPCs and samples can be dispensed using repeat dispense mode to save time and money.
- Plate layout of the blank, standards and samples in the WinKQCL software template is designed to offer the most flexibility for running full or partial plates.

Experimental set-up

Deck Position A: LAL water – 10 ml multichannel reagent reservoir
Deck Position B: PyroGene rFC Assay plate – 96 well flat bottom plate (Corning)
Deck Position C: Standards, samples and PPC – INTEGRA tube rack

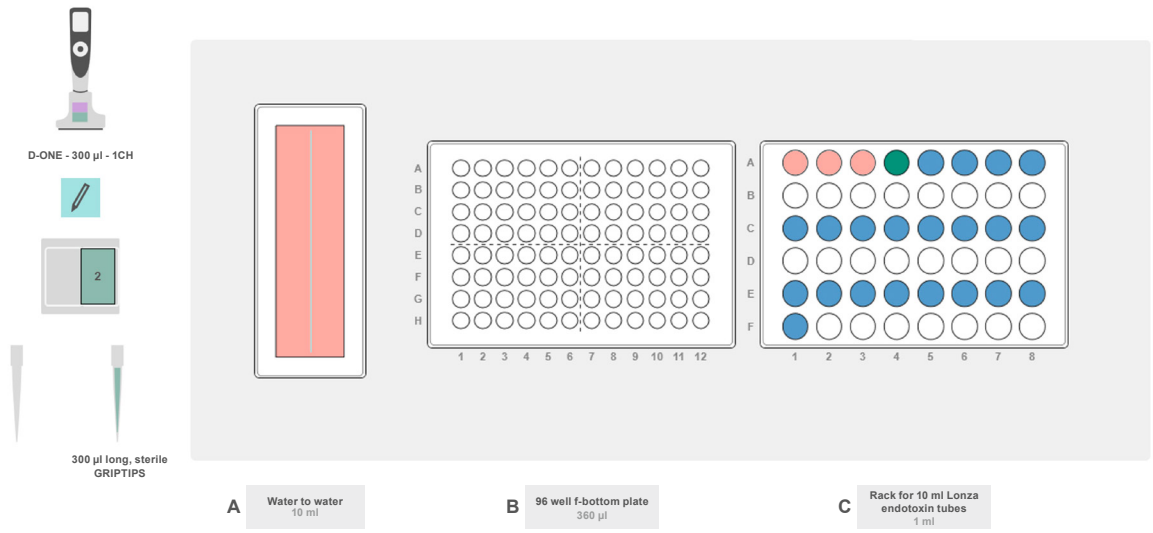


Figure 2: Set-up of the ASSIST PLUS for assay sample and standard addition. Position A: LAL water in 10 ml SureFlo reservoir. **Position B:** 96 well assay plate. Position C: Tube rack with Lonza pyrogen-free tubes (empty, magenta), 250 µl of 20 EU/ml endotoxin standard (green) and 1 ml of sample (blue).

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1. PyroGene rFC Assay plate set-up

STEP: Standards, samples and PPC-spiked samples are added to the plate.

HOW TO: Pair the 0.5-300 µl D-ONE single channel pipetting module with the ASSIST PLUS pipetting robot. Place pyrogen-free test tubes containing 1 ml sample in the tube rack on deck Position C (blue tubes in **Figure 2**). Place three empty tubes in Positions A1-A3 within the rack. These are used to create the standard dilutions (magenta tubes in **Figure 2**). Place a tube holding 250 µl of 20 EU/ml endotoxin standard in A4 in the rack (green tube in **Figure 2**). Place a 10 ml SureFlo reservoir holding LAL water on deck Position A, and a 96 well flat bottom plate on deck Position B.

When the 'Lonza PyroGene Assay plate set-up' program is started, the pipette first dispenses 900 µl of LAL water into each of the 3 empty dilution tubes (**Figure 3**). Next, 750 µl of LAL water is dispensed into the tube holding 250 µl of 20 EU/ml endotoxin standard, creating a 5 EU/ml standard. Following package insert instructions, a message on the pipette instructs the user to vortex the 5 EU/ml standard for 1 minute. The ASSIST PLUS pauses for the user to perform this step, then restarts when the user acknowledges the message. The next standard is created by transfer of 100 µl of the 5 EU/ml standard to the adjacent tube holding 900 µl of LAL water, followed by vortexing. The remaining 2 standards are created in a similar manner.

Figure 3: LAL water is dispensed by the D-ONE single channel pipetting module.

Once all the standards have been created, 10 µl of the 5 EU/ml standard is added to each well of the plate designated as a PPC (**Figure 4**). This serves as a control to monitor for sample inhibition of endotoxin detection. Each blank, standard and sample is then added to the appropriate wells in duplicate. Duplicate samples are also added to the PPC wells, creating the 0.5 EU/ml PPC-spiked samples. When all standards and samples have been added to the plate, the plate is pre-incubated at 37 °C for 10 minutes.

Figure 4: PPC is added by the D-ONE single channel pipetting module.

2. PyroGene rFC Assay reagent addition

STEP: Add 100 µl working reagent to each well of the plate.

HOW TO: While the plate is pre-incubating, manually prepare the working reagent in a 10 ml SureFlo reservoir by combining fluorogenic substrate, rFC assay buffer and rFC enzyme solution in a 5:4:1 ratio. Place the working reagent in a clean 10 ml SureFlo reservoir on deck Position A (**Figure 5**). Pair a 300 µl 8 channel VOYAGER pipette with the ASSIST PLUS, and exchange the D-ONE tip deck for a standard tip deck. At the end of the 10 minute pre-incubation, place the plate on deck Position B. Initiate the VIALAB program 'Lonza PyroGene Assay reagent addition' to add 100 µl reagent to each well of the plate. When reagent addition is complete, place the plate in the fluorescence microplate reader to complete the assay.

TIPS:

- VIALAB programs can be adapted to accommodate different numbers of samples, providing flexibility to meet current and future testing demands.
- For simplicity, 300 µl long GRIPTIPS are used in all steps of this assay.

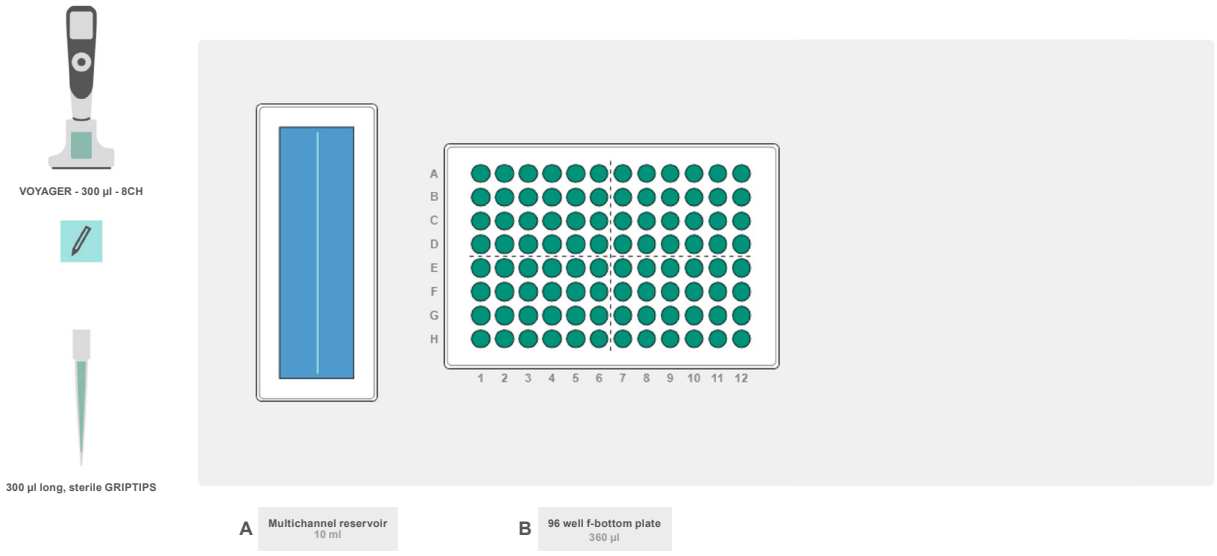

Figure 5: Deck set-up of the ASSIST PLUS for reagent addition. **Position A:** working endotoxin reagent in a 10 ml SureFlo reservoir. **Position B:** 96 well plate containing pre-incubated standards and samples.

Assay verification

Three runs of 21 samples plus standards were set up on the ASSIST PLUS, and tested according to the PyroGene rFC Assay instructions for use. Samples consisted of LAL water spiked with known concentrations of endotoxin standard. Five samples at each concentration were run on each plate, except for the 0 EU/ml sample, which was run 6 times per plate. Data analysis was performed using WinKQCL software. **Table 1** shows the concentrations of standards and samples.

Table 1: Endotoxin concentrations of standards and spiked samples.

Standards (EU/ml)	Samples of LAL water spiked with endotoxin (EU/ml)
5	2.5
0.5	0.25
0.05	0.025
0.005	0

Results

The results are displayed in **Tables 2** and **3**. The standard curve for all runs displayed good linearity, and all curves were within the quality parameters as defined in the instructions for use (**Table 2**). Samples spiked with each concentration of endotoxin were detected (**Figure 6**). All unspiked samples remained undetectable at <0.005 EU/ml, which is the cut-off for acceptable endotoxin concentrations in pharmaceuticals and medical devices. All replicates of standards, samples and PPCs displayed a CV within the acceptable limit of less than 25 % (**Table 3**).

Table 2: Standard curve results with quality specifications for runs 1-3.

	Correlation coefficient (0.980-1.000)	Slope (0.760-1.110)	Y-intercept (2.500-5.000)
Run 1	1.000	0.930	4.112
Run 2	0.999	0.903	4.258
Run 3	0.998	0.909	4.267

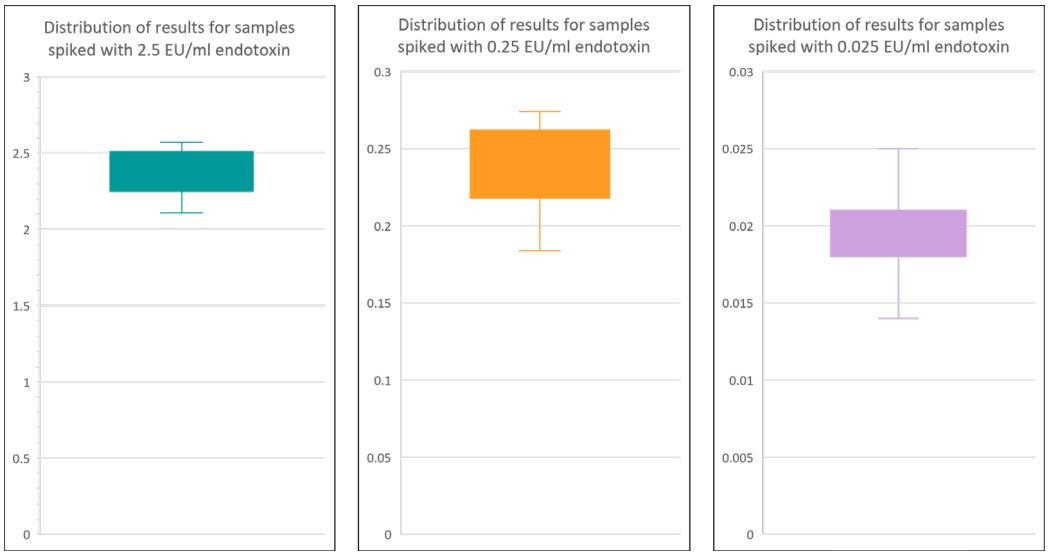

Figure 6: Distribution of results for samples spiked at endotoxin concentrations of 2.5, 0.25 and 0.025 EU/ml.

Table 3: Mean CV per plate for paired samples and PPCs.

	Mean paired sample %CV	Mean PPC %CV	QC fail %CV per plate
Run 1	4.19	4.37	0
Run 2	1.84	4.03	0
Run 3	3.66	3.87	0
Overall mean	3.23	4.09	0

Remarks

- **Partial plates:** The supplied VIALAB programs can be adapted for partial plates, or for running samples in triplicate.
- **Run report:** VIALAB programs can be started directly from a PC connected to the ASSIST PLUS pipetting robot. A report is automatically generated after the run, documenting details such as start and end times, user identification, calculated volumes and any errors that occurred. This offers a convenient way to fulfill regulatory requirements.

Conclusion

- The ASSIST PLUS provides an affordable, easy-to-use automation solution for low to medium throughput users of the Lonza PyroGene rFC Assay.
- High quality, reproducible results can be achieved with the ASSIST PLUS, eliminating the risk of costly and time-consuming retests.
- The ease and flexibility of VIALAB software allows users to customize plate layouts or set up partial plates using the same labware defined in this application note.

Materials

Manufacturer	Part Number	Description	Link
INTEGRA Biosciences	4505	ASSIST PLUS base unit	https://www.integra-biosciences.com/en/pipetting-robots/assist-plus
INTEGRA Biosciences	4531	0.5-300 µl D-ONE single channel pipetting module	https://www.integra-biosciences.com/en/pipetting-robots/assist-plus#parts-and-numbers
INTEGRA Biosciences	4723	300 µl 8 channel VOYAGER electronic pipette	https://www.integra-biosciences.com/en/pipetting-robots/assist-plus#parts-and-numbersrs
INTEGRA Biosciences	4535	Tip deck for D-ONE on ASSIST PLUS	https://www.integra-biosciences.com/en/pipetting-robots/assist-plus#parts-and-numbers
INTEGRA Biosciences	4552	6 x 8 tube rack	https://www.integra-biosciences.com/en/pipetting-robots/assist-plus
INTEGRA Biosciences	6484, specific lots	300 µl long, sterile (certified endotoxin free) GRIPTIPS	https://www.integra-biosciences.com/en/pipette-tips/grip-tip-selector-guide
INTEGRA Biosciences	4370, 4372, Specific lots	10 ml sterile polystyrene SureFlo Reagent Reservoir, certified endotoxin free	https://www.integra-biosciences.com/en/reagent-reservoirs/multichannel-reagent-reservoirs
Lonza	50-658U, 50-658NV	PyroGene Recombinant Factor C (rFC) Assay	https://bioscience.lonza.com/lonza_bs/CH/en/recombinant-factor-c-assay
Lonza	25-345S	Pyrowave XM Fluorescence Reader	https://bioscience.lonza.com/lonza_bs/US/en/Endotoxin-Detection/p/000000000000213090/PyroWave™-XM-Fluorescence-Reader
Lonza	25-611	WinKQCL Software	https://bioscience.lonza.com/lonza_bs/US/en/winkqcl-endotoxin-detection-and-analysis-software
Lonza	N207	Pyrogen-free Dilution Tubes	https://www.lonzabioscience.com.au/product/pyrogen-free-dilution-tubes-13x100mm-without-cap/
Lonza	W50-640	LAL Reagent Water	https://bioscience.lonza.com/lonza_bs/CH/en/Endotoxin-Detection/p/000000000000187031/LAL-Reagent-Water
Corning	3603	96 well, flat, clear bottom, black polystyrene, TC-treated microplate	https://ecatalog.corning.com/life-sciences/b2b/US/en/Microplates/Assay-Microplates/96-Well-Microplates/Corning®-96-well-Black-Clear-and-White-Clear-Bottom-Polystyrene-Microplates/p/3603
IKA		Vortex 2	https://www.ika.com/en/Products-LabEq/Shakers-pg179/Vortex-2-25000258/

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