Lonza

PyroGene®
Recombinant Factor C
Endotoxin Detection System

Catalog Number: 50-658U, 50-658NV

BLANK

TABLE OF CONTENTS

INTENDED USE

PyroGene® is intended for use as an *in vitro* end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices. This product is not intended for use in the detection of endotoxin in clinical samples or the diagnosis of human disease.

The PyroGene® test utilizes recombinant Factor C (rFC), an endotoxin- sensitive protein. rFC is used in combination with a fluorogenic substrate, an incubating fluorescence microplate reader and appropriate software to detect endotoxin. A minimum detection limit of 0.01 EU/ml and a measurable endotoxin concentration range of 0.01 to 10 EU/ml can be achieved.

WARNING

For *In Vitro* Diagnostic Use Only. PyroGene® is 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.

SAFETY PRECAUTIONS

The toxicological properties of the reagents supplied have not been tested. These reagents are not considered hazardous according to the OSHA Hazard Communication Standard 29 CFR 1910.1200. It is recommended that the NIH guidelines for recombinant DNA experiments be followed along with the use of standard laboratory precautions.

BACKGROUND

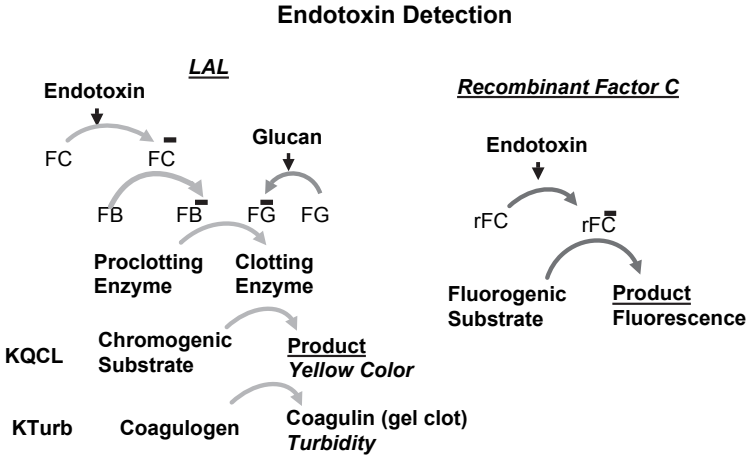
A Gram-negative bacterial infection of *Limulus polyphemus*, the horseshoe crab, may result in fatal intravascular coagulation.[1] At the molecular level, it has been demonstrated that endotoxin activates a serine protease catalytic coagulation cascade that results in the gelation of *Limulus* blood. This cascade is used in the Limulus Amebocyte Lysate (LAL) [1, 2, 3, 4] endotoxin detection method.

The protease cascade and rationale of traditional LAL tests are illustrated in the LAL pathway in Figure 1. Factor C, the first component in the cascade, is a protease zymogen that is activated by endotoxin binding[5, 6, 7]. In this pathway, Factor B (FB) is activated by Factor C. An alternative pathway, the Factor G (FG) pathway, can be activated by glucan binding[8]. Downstream, Factor C and Factor G pathways individually

activate a proclotting enzyme into a clotting enzyme. The chromogenic LAL assay (Lonza's Kinetic-QCL®, KQCL) uses a synthetic chromogenic peptide substrate that can be cleaved by the clotting enzyme, resulting in a product that exhibits a yellow color. The turbidimetric assay (Lonza's PYROGENT®-5000, KTurb) uses the native substrate, coagulogen, which can be cleaved into coagulin. Coagulin then begins to self-associate, resulting in an increase in turbidity. The densities of the yellow color (OD 405 nm) and turbidity (OD 340 nm) are correlated with endotoxin concentration.

Studies have demonstrated the ability of Factor C to selectively recognize endotoxin and activate the protease cascade. To create an endotoxin-specific assay, Factor C has been purified and cloned.[5,6,7,9,10,11] When activated by endotoxin binding, recombinant Factor C acts upon the fluorogenic substrate in the assay mixture to produce a fluorescent signal in proportion to the endotoxin concentration in the sample.

Figure 1. Schematic drawing of the endotoxin detection mechanisms in the LAL system and the rFC system.



PRINCIPLE

Recombinant Factor C is activated by endotoxin binding, and the active moiety created then acts to cleave a synthetic substrate resulting in the generation of a fluorogenic compound (Figure 1, rFC pathway). The assay is carried out in a 96-well plate. Fluorescence is measured at time zero and after a one-hour incubation at 37°C ± 1°C in a fluorescence microplate reader using excitation/emission wavelengths of 380/440 nm. The difference between the one-hour reading and the time zero reading (ΔRFU) is corrected for blank ΔRFU fluorescence. The log net fluorescence is proportional to the log endotoxin concentration and is linear in the 0.01-10 EU/ml range. Endotoxin in a sample is calculated relative to a standard curve.

STORAGE CONDITIONS

1. The PyroGene® kit, catalog numbers 50-658U, 50-658NV, is stored at 2-8°C.

REAGENTS SUPPLIED AND KIT CONFIGURATIONS

Catalog No. 50-658U contains:

1. Two vials of rFC Enzyme Solution, R50-658, 1.2 ml/vial
2. Two vials of the Fluorogenic Substrate, S50-658, 6.0 ml/vial
3. Two vials of the rFC Assay Buffer, B50-658, 5.0 ml/vial
4. Two vials of the *E. coli* Endotoxin, O55:B5, E50-643, lyophilized. The reconstitution volume of the vial is stated on the Certificate of Analysis included with each kit and is calculated to yield a solution containing 20 EU/ml. Reconstituted endotoxin is stable for 4 weeks at 2-8°C.
5. Two vials of LAL Reagent Water, W50-640, 30 ml/vial. This water is used to rehydrate *E. coli* endotoxin and to prepare endotoxin standard and sample dilutions.

Catalog No. 50-658NV contains:

1. Thirty vials of rFC Enzyme Solution, R50-658, 1.2 ml/vial
2. Thirty vials of the Fluorogenic Substrate, S50-658, 6.0 ml/vial
3. Thirty vials of the rFC Assay Buffer, B50-658, 5.0 ml/vial
4. Ten vials of the *E. coli* Endotoxin, O55:B5, E50-643, lyophilized. The reconstitution volume of the vial is stated on the Certificate of Analysis included with each kit and is calculated to yield a solution containing 20 EU/ml. Reconstituted endotoxin is stable for 4 weeks at 2-8°C.

MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

1. Disposable endotoxin-free glass dilution tubes (13 x 100 mm, #N207 or equivalent).
2. Individually wrapped sterile measuring pipettes.
3. Automatic hand-held pipettes with sterile, individually wrapped or racked tips.
4. Disposable sterile microplates. Note: Prior to routine use, microplates should be pre-qualified (#25-340 or equivalent).
5. Reagent reservoirs (#25-364 or equivalent).
6. Eight channel pipettor.
7. Fluorescence microplate reader (#25-344, FLx800™ TBIE reader with Ex 380/20 and Em 440/30).
8. Software (WinKQCL® V 3.0.1, or higher).
9. Timer.
10. Vortex Mixer.
11. For 50-658NV, LAL Reagent Water (#W50-640 or equivalent).

SPECIMEN COLLECTION AND PREPARATION

Careful technique must be used to avoid microbiological or endotoxin contamination. All materials coming in contact with the specimen or test reagents must be endotoxin-free. Clean glassware and materials may be rendered endotoxin-free by heating at 250°C for 30 minutes. Appropriate precautions should be taken to protect depyrogenated materials from subsequent environmental contamination.

From experience, most sterile, individually wrapped, plastic pipettes and pipette tips are endotoxin-free. However, these materials should be tested before regular use.

It may be necessary to adjust the pH of the sample to within the range 6.0-8.0 using endotoxin-free sodium hydroxide or hydrochloric acid. Always measure the pH of an aliquot of the bulk sample to avoid contamination by the pH electrode. Do not adjust unbuffered solutions.

Samples to be tested must be stored in such a way that all bacteriological activity is stopped or the endotoxin level may increase with time. For example, store samples at 2-8°C for less than 24 hours; samples stored longer than 24 hours should be frozen.

SENSITIVITY SETTING FOR FLUORESCENT READER

Fluorescence signals are normally recorded as Relative Fluorescence Units (RFU). Since the real fluorescence signal is converted to an electronic signal that can be "tuned" using the gain setting or sensitivity setting, RFU is an arbitrary unit. Depending on the strength of the detected signal, the instrument can be adjusted to a higher gain/sensitivity setting to boost a weak signal or tuned down to a lower gain/sensitivity setting when the signal is too strong. It is useful to consult the FLx800™ Reader Operator's Manual, Operation Section for the manufacturer's explanation.

In the PyroGene® assay, the 3-log endotoxin concentration range corresponds to the 3-log range in RFU. If the sensitivity is tuned too low, fluorescence at the lowest standard will be difficult to detect; if the sensitivity is tuned too high, fluorescence at the highest standard will be off-scale. Prior to conducting any assays, it is important to determine the appropriate sensitivity for the PyroGene® assay. The FLx800™ has a fluorescence range from 0 to 99999. In order to establish the 3-log fluorescence range for the standard curve, an RFU range from 1000 to 10000 was chosen for the 1 EU/ml standard. The RFU range from 1000 to 10000 corresponds to a log range from 3 to 4, with a log mid point of 3.5. Thus, the target absolute net RFU for the 1 EU/ml is approximately 3000 RFU. To maintain adequate separation between the blanks and the lowest standard in a routine assay, this value should be considered the minimum of a desirable range.

Determination of appropriate sensitivity setting

NOTE: This procedure should be conducted as part of the performance qualification for any new lot of reagents, after major equipment maintenance (such as lamp replacement) or when reader performance is questioned. A periodic re-verification is recommended as part of any on-going equipment maintenance program.

1. Prepare a 1 EU/ml endotoxin solution (see Preparation of Endotoxin Standards). Allow reagents to equilibrate to ambient temperature before mixing.

2. Using WinKQCL® Software, select *Validation/FLx800 Reader/SensitivityTest* then click the *New* button.

3. Enter appropriate information in blank fields and confirm settings for the test.

Endotoxin Concentration Unitage	EU
Time Between Reads (hh:mm:ss)	01:00:00
Excitation Filter (nm)	380:20
Emission Filter (nm)	440:30
Reads per Well	4
Delay before reading (millisec.)	150
Optics Position	bottom
Wells to Use: Pre-defined	(D6,7; E6,7; F6,7)
Sensitivity (select by checking)	30, 35, 40, 45, 50, 55

4. Add 100 µl of the 1 EU/ml solution to wells D6,7; E6,7; F6,7.

5. Pre-incubate covered plate with standards at 37°C ± 1°C for 10 minutes.

6. Prepare working reagent by mixing rFC enzyme solution, rFC assay buffer and fluorogenic substrate at 1:4:5 ratios, respectively. 100 µl + 400 µl + 500 µl is a sufficient quantity for the sensitivity test. Refrigerate any unused reagents after opening. Uncontaminated previously opened vials remain effective throughout the lifetime of the reconstituted endotoxin if returned to refrigeration. NOTE: The order of mixing should remain consistent. Add enzyme last to the combined buffered substrate. Mix thoroughly but gently. Avoid vortexing of the working reagent mix. NOTE: Once prepared, the working reagent cannot be stored.

7. At the appropriate software prompt add 100 µl of working reagent to all wells that contain the endotoxin sample. Click OK to initiate test. Note: leave the plate uncovered for the duration of the test.

8. At the completion of the test a summary of the results with the calculated sensitivity will be displayed. Using linear regression, the sensitivity value correlated to log ΔRFU = 3.5 is rounded to the nearest integer. Successful assays should result when using this setting as a minimum. Both the *Analyst* and *Reviewer* should e-sign then print the report.

Sensitivity Setting Determined:_____
for Kit Lot :_____

PREPARATION OF ENDOTOXIN STANDARDS

In order to calculate the endotoxin concentration in unknown samples, each PyroGene® test must be referenced to a valid standard curve.

Because of the large concentration range over which endotoxin values can be determined, it is possible to adjust the quantitative range of any given test by adjusting the concentration of endotoxin standards used to generate the standard curve. A minimum of three standards is required.

The PyroGene® assay has been optimized to be linear from 0.01 EU/ml to 10 EU/ml. However, the individual user may choose to truncate the standard curve depending on specific product testing requirements.

The *E. coli* endotoxin, O55:B5, is provided for user convenience. Other endotoxin preparations may be used to prepare the standards; however, their performance in the PyroGene® assay relative to the current USP Reference Standard Endotoxin (RSE) must be determined.

The supplied lyophilized *E. coli* O55:B5 endotoxin is reconstituted with LAL Reagent Water to yield a 20 EU/ml stock solution. The reconstitution volume of the vial is stated on the Certificate of Analysis, included with each kit, and is calculated to yield a solution containing 20 EU/ml. Reconstitute with the specified volume of LAL Reagent Water. Shake vigorously for at least 15 minutes at high speed on a vortex mixer. Prior to future use, the solution must be warmed to room temperature and vigorously vortexed for 15 minutes.

The following table suggests a dilution scheme for constructing a series of endotoxin dilutions from the endotoxin supplied in the kit.

Endotoxin Concentration (EU/ml)	Volume of LAL Reagent Water	Volume of Endotoxin Standard added to LAL Reagent Water
10	0.5 ml	0.5 ml of 20 EU/ml solution
1	0.9 ml	0.1 ml of 10 EU/ml solution
0.1	0.9 ml	0.1 ml of 1 EU/ml solution
0.01	0.9 ml	0.1 ml of 0.1 EU/ml solution

1. Prepare a solution containing 10 EU/ml endotoxin by adding 0.5 ml of the 20 EU/ml endotoxin stock solution into 0.5 ml of LAL Reagent Water in a suitable container. This solution should be vigorously vortexed for at least 1 minute before proceeding.

2. Transfer 0.1 ml of the 10 EU/ml endotoxin solution into 0.9 ml of LAL Reagent Water in a suitable container and label 1 EU/ml. The solution should be vigorously vortexed for at least 1 minute before proceeding.

3. Transfer 0.1 ml of the 1 EU/ml endotoxin solution into 0.9 ml of LAL Reagent Water in a suitable container and label 0.1 EU/ml. The solution should be vigorously vortexed for at least 1 minute before proceeding.

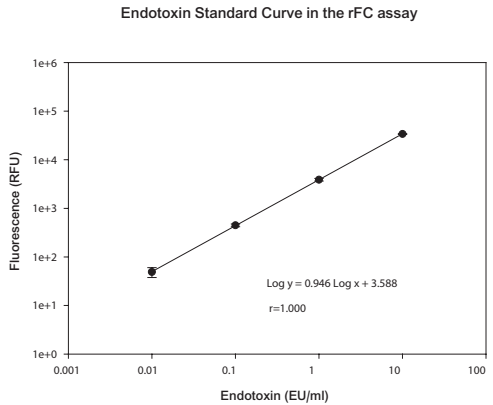
4. Transfer 0.1 ml of the 0.1 EU/ml endotoxin solution into 0.9 ml of LAL Reagent Water in a suitable container and label 0.01 EU/ml. The solution should be vigorously vortexed for at least 1 minute before proceeding.

TESTING PROCEDURE

General testing procedure

1. The PyroGene® endotoxin assay uses a 96-well plate assay format. Add 100 µl of the blank, endotoxin standards, and samples to appropriate wells of the microplate. Duplicate or triplicate assays are generally used.
2. To spike the samples with endotoxin, add 10 µl of the 1 EU/ml solution to the designated Positive Product Control wells.
3. Pre-incubate the plate in the reader at 37°C ± 1°C for a minimum of 10 minutes.
4. During the incubation period, prepare the working reagent. The rFC enzyme solution, assay buffer and substrate are mixed in a 1:4:5 ratio, respectively, to form the working reagent.

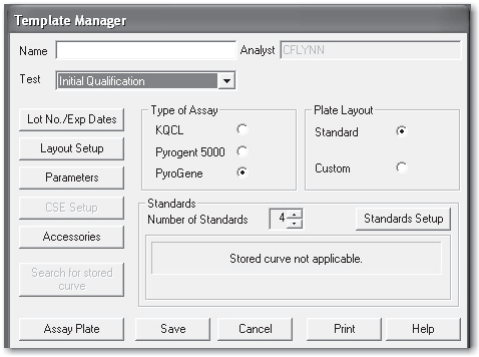
5. Carefully dispense 100 µl of the working reagent to each well.
6. Read fluorescence at time zero.
7. Incubate the reaction for one hour and read the plate at elapsed time one-hour.
8. The difference of time one-hour and time zero readings are corrected with the blank.
9. The log net ΔRFU is then plotted against log endotoxin concentration in a linear regression curve.
10. An example curve is shown below:



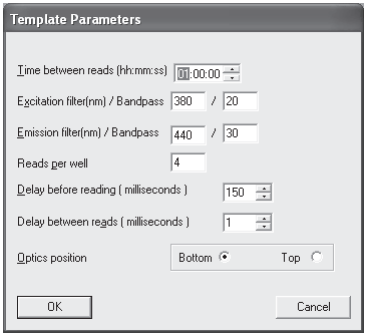
11. Calculate endotoxin concentration of samples according to the standard curve.

When using FLx800™ and WinKQCL® Software

The fluorescence reader FLx800™ and WinKQCL® Software can be used in this assay. Turn on the reader and launch the software. In the software, create a template for the rFC assay using *Templates / New*. Several types of assays may be programmed. Consult the Software User Manual for more information. The example that follows describes setting up an Initial Qualification Assay.

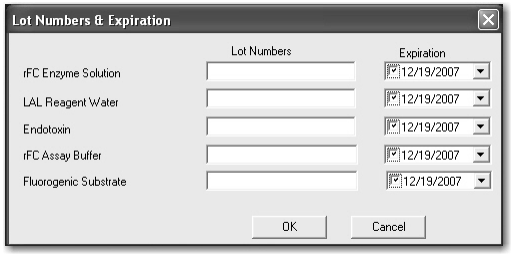


1. Name: Enter the template name. The template name can be a maximum of 20 characters long, and no two templates can have the same name.
2. Analyst: The Analyst ID of the user is automatically entered and cannot be changed.
3. Test Type: Click on the down arrow button then click on Initial Qualification.
4. Type of Assay: Select the PyroGene® radio button.
5. Parameters: The reader parameter values for a template may be verified or changed by clicking on Parameters.



Settings pictured are the defaults used in the assay.

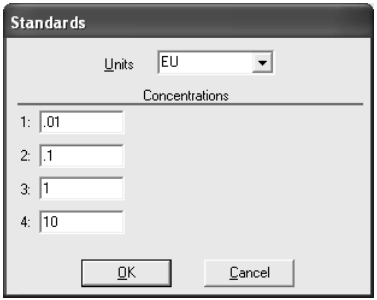
6. Lot Numbers: Selecting the Lot No./Exp Dates button will bring up the following screen:



Enter all appropriate information.

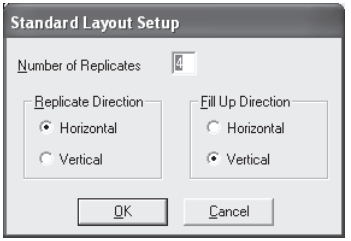
7. Standards: Select the number of standards by clicking on the up or down arrow button. A minimum of 3 and a maximum of 12 standards are allowed. Four standards are typically run with the PyroGene® assay.

8. Standards Setup: Click on Standards Setup to enter the concentrations and units of the standard curve.



Click OK after completing the Standards Setup dialog box.

9. Layout:



Defines the positioning of the blank, standards and samples. Number of replicates is selectable from 2 to 6. Click OK after completing the Standard Layout Setup dialog box.

10. Save the template. From the list of the templates an assay is initiated by selecting Run. Follow on screen prompts to begin the test. Consult Software User Manual for more details.

11. Place the microplate (loaded with blank and standards) in the reader and pre-incubate the plate for a minimum of 10 minutes at 37°C ± 1°C.

12. During the 10 minute incubation, prepare working reagent by mixing the rFC enzyme solution, the assay buffer and the fluorogenic substrate in a 1:4:5 ratio, respectively. To ensure enough working reagent is prepared for the assay and excess is minimal, determine the number of wells requiring working reagent, add 4 additional wells, and calculate the appropriate amount for each component to make the working reagent. Allow reagents to equilibrate to room temperature before mixing. The order of mixing should remain consistent. Add enzyme last to the combined buffered substrate. Mix gently but thoroughly. Do not vortex. Refer to the following table:
NOTE: Once prepared, the working reagent cannot be stored.

Volume (µl) of reagent required per total number of wells				
	To assure slight excess, assume volume prepared is sufficient for an additional 4 wells			
Total number of wells	rFC enzyme solution	rFC assay buffer	Fluorogenic substrate	Total Volume
6	100	400	500	1000
12	160	640	800	1600
24	280	1120	1400	2800
36	400	1600	2000	4000
42	460	1840	2300	4600
48	520	2080	2600	5200
54	580	2320	2900	5800
60	640	2560	3200	6400
66	700	2800	3500	7000
72	760	3040	3800	7600
78	820	3280	4100	8200
84	880	3520	4400	8800
90	940	3760	4700	9400
96	1000	4000	5000	10000

13. When prompted by the software at the end of the incubation period, dispense 100 µl of working reagent into the appropriate wells using an eight channel pipettor.

14. Immediately click *OK*. The software will initiate read one (time zero reading) immediately and read two an hour later.

15. After the assay is completed, the data is automatically saved to the hard drive.

TYPES OF PYROGENE® ASSAYS

The incubating fluorescence microplate reader and WinKQCL® Software are an integral part of the PyroGene® assay. It is important to become familiar with the operation of the incubating microplate reader and the features of the WinKQCL® Software. Please refer to the incubating microplate reader and WinKQCL® Software Manuals for more detailed information.

There are four (4) basic types of PyroGene® assays. Each type is designed to perform a different aspect of endotoxin testing.

1. ROUTINE

A ROUTINE assay calculates the concentration of endotoxin in unknowns by comparison to the performance of a series of endotoxin standards.

As part of a ROUTINE assay, the user has the option to include a Positive Product Control (PPC) as a monitor for product inhibition or enhancement. A PPC is a sample of product to which a known amount of endotoxin spike has been added. The WinKQCL® Software automatically calculates the amount of endotoxin recovered in the PPC, allowing for a comparison to the known amount of endotoxin spike.

2. INHIBITION/ENHANCEMENT (INH/ENH)

The PyroGene® reaction is enzyme mediated and, as such, has an optimal pH range and specific salt and divalent cation requirements. Occasionally, test samples may alter these optimal conditions to an extent that the rFC is rendered insensitive to endotoxin. Negative results with samples which inhibit the PyroGene® test do not necessarily indicate the absence of endotoxin.

An INHIBITION/ENHANCEMENT assay is designed to determine what level of product dilution overcomes inhibition or enhancement. Each product dilution must be accompanied by a Positive Product Control (PPC). The WinKQCL® Software calculates the amount of endotoxin recovered in the PPC for comparison to the known amount of endotoxin spike. In this manner it can be determined which product dilutions are non-inhibitory.

3. RSE/CSE

An RSE/CSE assay is designed to determine the potency of a Control Standard Endotoxin (CSE) in terms of the concentration units of the Reference Standard Endotoxin (RSE).

The assay requires a single series of RSE dilutions and one or more sets of dilutions of the CSE. Depending on the concentration units of the CSE, the WinKQCL® Software automatically computes mean potency values in terms of EU/ml or EU/ng. The user also has the option to enter units other than EU or ng.

4. INITIAL QUALIFICATION (INT. QUAL)

An INITIAL QUALIFICATION assay is designed according to the requirements described in the FDA's "Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices".[12] This assay is required as part of the validation of the LAL assay and is also to be performed with each new kit lot. PyroGene® testing evolved from the LAL test and Lonza suggests a similar approach be utilized.

The INITIAL QUALIFICATION assay performs a log/log linear correlation of the individual Net ΔRFU values for each replicate of each endotoxin standard. The other assays use the average Net ΔRFU of all the replicates of each standard.

The INITIAL QUALIFICATION assay does not provide for the inclusion of any samples.

PERFORMANCE CHARACTERISTICS

Linearity*

The linearity of the standard curve within the concentration range used to determine endotoxin values should be verified. Assay no less than 3 endotoxin standards, spanning the desired concentration range, and a LAL Reagent Water blank at least in triplicate. Additional standards should be included to bracket each log interval over the range of the standard curve. The value of the correlation coefficient (r) of the calculated standard curve should be ≥ 0.980.

*See Further data considerations under Other Technical Issues section.

PRODUCT INHIBITION

Product inhibition occurs when substances in the test sample interfere with the enzyme reaction. Inhibition results in a lower final ΔRFU in a sample reading, indicating lower levels of endotoxin than what may actually be present in the test sample. The lack of product inhibition should be determined for each specific sample, either undilute or at an appropriate dilution.

To verify the lack of product inhibition, an aliquot of a test sample (or a dilution of a test sample) is spiked with a known amount of endotoxin. It is recommended that the endotoxin spike result in a final endotoxin concentration in the sample equal to 0.1 EU/ml. For samples which may contain a background endotoxin level > 0.1 EU/ml, the endotoxin spike should result in a final endotoxin concentration of 1.0 EU/ml. The spiked solution is assayed along with the unspiked samples and their respective endotoxin concentrations are determined. The differences between these two calculated endotoxin values should equal the known concentration of the spike within the range of 50-200%.[13]

A spiked aliquot of the test sample (or dilution) may be prepared as in one of the following examples:

Tube Method:

Transfer 50 µl of the 10 EU/ml solution into 4.95 ml of test sample (or dilution). This solution contains an endotoxin concentration of 0.1 EU/ml in test sample (or dilution). This sample should be vigorously vortexed for one minute prior to use.

Plate Method #1:

Transfer 10 µl of the 1 EU/ml solution into each of the PPC wells in the 96-well plate, as directed by the assay template. To these wells add 100 µl of test sample (or dilution). Each well will now contain a 0.1 EU/ml solution. Mix gently.

Plate Method #2:

Place 100 µl of test sample (or dilution) into the PPC wells in the 96-well plate, as directed by the assay template. To these wells, add 10 µl of the 1 EU/ml solution. Each well will now contain a 0.1 EU/ml solution. Mix gently.

If the test sample (or dilution) is found to be inhibitory to the enzyme reaction, the sample may require further dilution until the inhibition is overcome.

Determination of a Non-Inhibitory Dilution with 0.1 EU/ml Endotoxin Spike

Sample Dilution	Raw Endotoxin (EU/ml)			Spike Recovery	Sample Inhibition	Endotoxin (Raw x DF)
	Unspiked	Spiked	Difference			
1/10	0.075	0.100	0.025	25%	Inhibitory	N/A
1/20	0.044	0.088	0.044	44%	Inhibitory	N/A
1/40	0.025	0.105	0.080	80%	Non-Inhibitory	1 EU/ml

Initially, Lonza recommends screening for product inhibition by testing ten-fold dilutions of the test sample. Once the approximate non-inhibitory dilution is determined, the exact dilution can be found by testing two-fold dilutions around this dilution.

REFERENCES

1. Levin, J. and F.B. Bang. The Role of endotoxin in the extracellular coagulation of *Limulus* blood. *Bull. Johns Hopkins Hosp.* 115: 265 (1964).
2. Levin, J. and F.B. Bang. A description of cellular coagulation in the *Limulus. Bull. Johns Hopkins Hosp.* 115: 337 (1964).
3. Levin, J. and F. B. Bang. Clottable protein in *Limulus*: its localization and kinetics of its coagulation by endotoxin. *Thromb. Diath. Haemorrh.* 19: 186 (1968).
4. Iwanaga S. The *Limulus* clotting reaction. *Curr. Opin. Immunol.* 5: 74 (1993).
5. Nakamura T., Morita T., Iwanaga S. Lipopolysaccharide-sensitive serine-protease zymogen (factor C) found in *Limulus* hemocytes. *Eur. J. Biochem.* 154: 511 (1986).
6. Muta T., Miyata T., Misumi Y. et al. *Limulus* Factor C: an endotoxin-sensitive serine protease zymogen with a mosaic structure of complement-like, epidermal growth factor-like, and lectin-like domains. *J. Biol. Chem.* 266: 6554 (1991).
7. Tokunaga F., Nakajima H., Iwanaga S. Further studies on lipopolysaccharide-sensitive serine protease zymogen (factor C): its isolation from *Limulus polyphemus* hemocytes and identification as an intracellular zymogen activated by alpha-chymotrypsin, not by trypsin. *J. Biochem.* 109: 150 (1991).
8. Morita, T. et al. A new (1-3) beta-D glucan mediated coagulation pathway found in *Limulus* amebocytes. *FEBS Letts.* 129: 318 (1981).
9. Ding J.L., Navas M.A.A., Ho B. Two forms of Factor C for the amebocytes of *Carcinoscorpius rotundicauda:* purification and characterization. *Biochim. Biophys. Acta* 1202: 149 (1993).
10. Ding J.L., Navas M.A.A., Ho B. Molecular cloning and sequence analysis of Factor C cDNA from the Singapore horseshoe crab, *Carcinoscorpius rotundicauda. Mol. Mar. Biol. Biotechnol.* 4: 90 (1995).
11. Ding J.L. and Ho B. New era in pyrogen testing. *Trends in Biotechnology,* 19: 277 (2001).
12. U.S. Department of Health and Human Service, Food and Drug Administration, "Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices" (1987).
13. Chapter <85> Bacterial Endotoxins Test. Rockville, MD: United States Pharmacopeia.

PATENT INFORMATION

Various components of PyroGene® are protected under the following patents:
US 4,745,051; US 4,879,236; US 5,716,834; US 5,712,144; Australia 581,174; Canada 1,222,213; Colombia 24,556; Denmark 172,401; EP 127,839; Ireland 58,011; Israel 71,906; Japan 2,129,487 & 2,644,447; Korea 51,077; Mexico 164,250; Norway 173,944; New Zealand 208,259; Philippines 25,395; South Africa 843,914; Spain 532,825; Taiwan 50,740. Additional Patents Pending.

OTHER TECHNICAL ISSUES

Improving spike recovery

By using a different data treatment procedure (for example, a polynomial curve fit using POWERCURVE™), calculation of the recovery of the positive product control can be improved.

Truncation of standard curve

In our experience, a truncated range of endotoxin standards, 0.01 – 1 EU/ml or 0.1-10 EU/ml, can provide better linearity than the whole range of 0.01- 10 EU/ml.

Overcoming inhibition with a dispersing agent

PYROSPERSE®, #N190, a dispersing agent, is not compatible with the PyroGene® assay.

TRADEMARK INFORMATION

NOTES

NOTES

Lonza

8830 Biggs Ford Road | Walkersville, MD 21793
301-898-7025 | www.lonza.com

08299
P50-658U/NV-6
12/07