PyroGene®
Recombinant Factor C
Endotoxin Detection System

Catalog Number: 50-658U, 50-658NV
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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. 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) 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. 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. 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. 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).
11. For 50-658NV, LAL Reagent Water (#W50-640 or equivalent).
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 \( \Delta RFU = 3.5 \) 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.

<table>
<thead>
<tr>
<th>Endotoxin Concentration (EU/ml)</th>
<th>Volume of LAL Reagent Water</th>
<th>Volume of Endotoxin Standard added to LAL Reagent Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.5 ml</td>
<td>0.5 ml of 20 EU/ml solution</td>
</tr>
<tr>
<td>1</td>
<td>0.9 ml</td>
<td>0.1 ml of 10 EU/ml solution</td>
</tr>
<tr>
<td>0.1</td>
<td>0.9 ml</td>
<td>0.1 ml of 1 EU/ml solution</td>
</tr>
<tr>
<td>0.01</td>
<td>0.9 ml</td>
<td>0.1 ml of 0.1 EU/ml solution</td>
</tr>
</tbody>
</table>

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 \( \mu l \) 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 \( \mu l \) 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:

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.

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

![Lot Numbers & Expiration](image)

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.

![Standards](image)

Click OK after completing the Standards Setup dialog box.

9. Layout:

![Standard Layout Setup](image)

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.
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”. 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.

<table>
<thead>
<tr>
<th>Volume (µl) of reagent required per total number of wells</th>
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<tbody>
<tr>
<td>To assure slight excess, assume volume prepared is sufficient for an additional 4 wells</td>
</tr>
<tr>
<td>Total number of wells</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>24</td>
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<td>36</td>
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<td>72</td>
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<td>78</td>
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<td>84</td>
</tr>
<tr>
<td>90</td>
</tr>
<tr>
<td>96</td>
</tr>
</tbody>
</table>

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.
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.

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.
PATENT INFORMATION

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

The FLx800™ is a trademark of BioTek Instruments, Inc.

Unless otherwise noted, all trademarks herein are marks of the Lonza Group or its affiliates.