Recombinant LAL Reagent

PyroSmart NextGen™

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PyroSmart NextGen™

Recombinant Kinetic Chromogenic Reagent for the Detection and Quantification of Gram Negative Bacterial Endotoxins (Lipopolysaccharides)

Intended Use
The PyroSmart NextGen™ recombinant assay may be used as an alternate test to compendial testing for the end-product testing of human injectable drugs (including biological products), animal injectable drugs, and medical devices (1,2). Guidance on validation of alternate test methods can be found in USP <1223> and <1225> (3,4) and such methods should be shown to be equivalent or superior to compendial methods. This assay may be also used for the quantitation of endotoxin in non-compendial articles (e.g. raw materials, including water, and for in-process monitoring) without method validation.

The PyroSmart NextGen™ recombinant assay is not intended for use in the detection of endotoxin in clinical samples for the diagnosis of human disease such as endotoxemia in humans.

Test Principle
PyroSmart NextGen™ reagent consists of three recombinant proteins: Factor C, Factor B and Proclotting Enzyme. In the presence of endotoxin, recombinant Factor C becomes an activated moiety which in turn activates recombinant Factor B and recombinant Proclotting Enzyme; ultimately resulting in the proteolytic cleavage of a colorless chromogenic substrate formulated with PyroSmart NextGen™. Cleavage of the substrate liberates *para*-nitroaniline (pNA), which is yellow and absorbs at 405 nm (Figure 1). The change in absorbance is continuously measured at regular intervals at 37 ± 1°C during an appropriate runtime. The greater the endotoxin concentration, the faster the pNA releases resulting in a faster change in absorbance.

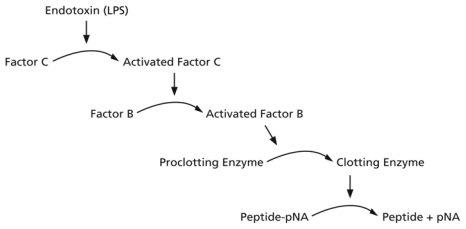

Figure 1 Cascade mechanism starting with endotoxin-activation of Factor C and yielding absorbance increase as a result of pNA release

Safety Precautions
The toxicity of PyroSmart NextGen™ has not been determined. Thus, caution should be exercised when handling PyroSmart NextGen™.

Storage Conditions
Date of Expiry is stated on the vial and external packaging.

Table 1: Storage conditions for PyroSmart NextGen™

Lyophilized Reagent	Store at 2-8°C
Reconstitution Buffer	Store at 2-8°C. Keep at room temperature at least 30 min before testing
Reconstituted Reagent	Should be used immediately after reconstitution (within 20 min)

Assay Conditions
PyroSmart NextGen™ can be used to quantify endotoxin concentration in two ways:

1. *Onset Time Assay:* where the time taken to reach a threshold OD (referred to as onset time) is determined. Higher endotoxin concentrations give shorter onset times. The standard curve is constructed by plotting the log onset time (Y-axis) against the log standard concentration (X-axis) and is used to calculate endotoxin concentrations in samples.
2. *Rate Assay:* where the mean rate (Vmean: mAbs/ min) is calculated over the course of the test. Higher endotoxin concentrations give higher Vmean values. The standard curve is constructed by plotting Vmean (Y-axis) against the standard concentration (X-axis) and is used to determine endotoxin concentrations in samples.

The software settings for both assays are summarized in Table 2.

Table 2: Software Settings for PyroSmart NextGen™ Assays

	Onset Time Assay	Rate Assay
Shake	10 sec	10 sec
Read	Kinetic, Absorbance	Kinetic, Absorbance
Wavelength	405nm	405/490nm*
Reading Interval	30 sec**	30 sec**
Runtime	60 min	30 min
Data Reduction	Onset OD = 0.03 OD	Pyros® eXpress: Vmean Gen5™: Mean V SoftMax® Pro : Vmax

*Or 405/492nm depending on the ability of the plate reader

**Interval may vary based on plate reader

Materials and Equipment
Materials supplied with PyroSmart NextGen™ are listed in Table 3. Materials and equipment required but not supplied with PyroSmart NextGen™ are listed in Table 4.

Table 3: Material Supplied with PyroSmart NextGen™

Component	No. of Vials	Notes
PyroSmart NextGen™ Reagent	2	Reconstitute each vial with 2.8mL of reconstitution buffer
PyroSmart NextGen™ Reconstitution Buffer	2	-

Table 4: Materials & Equipment Required but NOT Supplied with PyroSmart NextGen™

Equipment Type	Specification	Description/Catalog No.+
Incubating absorbance platereader	Capable of maintaining a temperature of 37°C while collecting absorbance reads	e.g. BioTek® ELx808™, Molecular Devices readers or equivalent
Platereader software	Allows for data reduction by Onset Time or Rate	e.g. Pyros® eXpress or Gen5™ for ELx808™, Softmax® Pro for Molecular Devices readers; or equivalent
Control Standard Endotoxin (CSE)++	10ng/vial calibrated against RSE with PyroSmart NextGen™	e.g. ACC EC010-5 or equivalent
LAL Reagent Water (LRW)	Free of interfering endotoxins	e.g. ACC WP050C or equivalent
96-well Microplates	Covered, non-coated, untreated microplates, free of interfering endotoxin	e.g. ACC CA961-10 or equivalent
Depyrogenated Glass Dilution Tubes	Free of interfering endotoxin, should not interfere with the test	e.g. ACC TB240-5, TB013-5, TB016C or equivalent
A set of adjustable single-channel micropipettes	Capable of delivering volumes of 5-20uL, 20 – 100uL and 100-1000uL	Gilson, Rainin traditional or Eppendorf model fit the tips below or equivalent
Pipette tips	Free of interfering endotoxin Capable of delivering volumes of: 5-20uL, 20 – 100uL and 100-1000uL	e.g. ACC PPT25, PPT10 or equivalent
Repeating pipette with compatible syringe barrels	Auto-delivery of aliquots	e.g. Eppendorf Xstream® repeater with BioPur® combi-tip 2.5mL or equivalent
Vortex mixer	Any	Any
Timer	Any	Any
Parafilm M®	The side in contact with the paper backing is typically free of detectable endotoxin.	American National Can™
Tube rack	Any	Any
Slanted plate stand	Any	Any

+Note: Not all products are available globally. Refer to your local supplier.

++Note: The Certificate of Analysis and the potency stated on it are specific to a combination of PyroSmart NextGen™ and CSE lot. A given lot of CSE may show different potencies (EU/ng) when tested with different lots of PyroSmart NextGen™. Similarly, different lots of CSE will likely have different potencies when tested with the same lot of PyroSmart NextGen™.

Controls
Negative Control: LAL Reagent Water (LRW) serves as a negative control.

Standard Curve: A standard curve series as a geometric series should yield the range of endotoxin concentrations required. For examples, refer to Table 5.

Table 5: Examples of Standard Curve Ranges and Setups for Both Assays

Onset Time Assay		
CSE (or RSE) concentration in EU/mL	Volume of LRW	CSE (or RSE) solution in EU/mL
50	-	-
5	900µL	100µL of 50 EU/mL
0.5	900µL	100µL of 5 EU/mL
0.05	900µL	100µL of 0.5 EU/mL
0.005	900µL	100µL of 0.05 EU/mL
Rate Assay		
CSE (or RSE) concentration in EU/mL	Volume of LRW	CSE (or RSE) solution in EU/mL
0.1	1960µL	40µL of 5 EU/mL
0.05	500µL	500µL of 0.1 EU/mL
0.025	500µL	500µL of 0.05 EU/mL
0.0125	500µL	500µL of 0.025 EU/mL
0.00625	500µL	500µL of 0.0125 EU/mL

Positive Product Controls (PPC): PPCs are suitability (inhibition/enhancement) controls and consist of a sample (or dilution of the sample) to which standard endotoxin is added. The added endotoxin should yield a concentration that falls in the middle of the standard curve. For example, if the standard curve is 50 to 0.005 EU/mL, spike the 50µL of sample with 5uL of 5 EU/mL to render a final concentration of 0.5 EU/mL. If the standard curve is 0.1 to 0.00625 EU/mL, spike the 50µL of sample with 5µL of 0.5 EU/mL to render a final concentration of 0.05 EU/mL.

Test Procedure

1. Turn on the plate reader to allow equilibrating at 37°C.
2. Set up the software using appropriate settings (see Table 2).
3. Prepare the appropriate controls.
4. Prepare the microplate as shown in Figure 2. The microplate setup is described in more detail below.
5. Read the test.

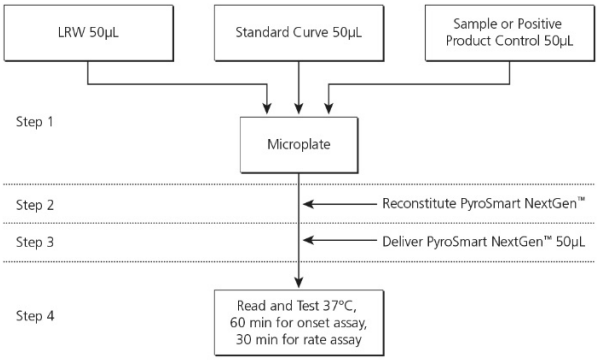

Figure 2: Preparation of Microplate

STEP 1: Transfer Test Sample
Transfer 50uL of test sample (negative control x2, endotoxin standard series x2, sample dilutions x2 and PPC for each sample dilution x2) to the appropriate wells of the microplate as defined in software plate layout.

STEP 2: Reconstitute PyroSmart NextGen™ Recombinant LAL Reagent
Gently tap the vial to cause loose material to fall to the bottom. Break the vacuum by aseptically lifting the stopper. Discard the stopper. Transfer 2.8 mL of PyroSmart NextGen™ Reconstitution Buffer to the reagent vial and cover with Parafilm. Allow the pellet to go fully into solution for a minimum of 3 minutes prior to use. Immediately before use, swirl the vial to insure homogeneity but avoid vigorous mixing that may cause excessive foaming and a loss of sensitivity. Use immediately within 20 min after reconstitution.

STEP 3: Deliver PyroSmart NextGen™ to the microplate
Remove the plate cover. Use the repeater pipette set to deliver 50uL aliquots, one aliquot at a time. Avoid cross-contamination by using the pipette under a 45 degree angle to dispense the reagent to the side of the well. Start with adding to negative controls, followed by lowest standard concentration across to the highest, and finally all samples. Proceed as rapidly as possible (no longer than 30 seconds). Replace the plate cover.

STEP 4: Read the test
Transfer the microplate into a plate reader. Remove the plate cover and close the reader. Start the test.

Assay Run Validity Criteria
In order for the run to be valid, the conditions listed in Table 6 have to be met.

Table 6: Examples of Standard Curve Ranges and Setups for Both Assays

Criteria	**Validity**
Negative Control	*Onset Time Assay*: The onset time of negative controls must be greater than that of the lowest standard concentration. *Rate Assay:* The Vmean of negative control must be lower than that of the lowest standard concentration. It should be less than or equal to 1.0 mAbs/min.
Standard Curve	The standard curve must have an absolute value of a correlation coefficient ≥ 0.980.
Positive Product Controls	The recovery of the positive product control must be within 50 to 200% of the nominal concentration of the added endotoxin.

Results
All the calculations described in this section are automatically performed by appropriately configured software. Contact Technical Services at techservice@acciusa.com for further assistance.

Calculation the endotoxin concentrations
Interpolate endotoxin concentrations of all test samples (including standards and controls) using the equation for a straight line Y = Slope * X + Y-intercept (for Onset Time assay: Y = log onset time and X = log endotoxin concentration, for Rate assay: Y = Vmean and X = endotoxin concentration) rearranged as:

- *Onset Time Assay:* Log Endotoxin Concentration = (Log Onset Time – Y-intercept) / Slope
- *Rate Assay:* Endotoxin Concentration = (Vmean – Y-intercept) / Slope

PPC recovery for spiked samples
PPC recovery % = (Mean concentration in spiked sample – Mean concentration in unspiked sample) / nominal spike concentration x 100%

Final endotoxin concentration in unspiked samples
Multiply the endotoxin concentration found in the diluted sample by the dilution factor to express the concentration in the original sample before dilution.

Limitations of the Procedure
The procedure is limited by the extent of the inhibition or enhancement capacity of the sample under test. Substances that denature proteins, chelate ions, bind endotoxin or alter endotoxin's hydrophobic state may interfere with the test. Interference may be detected as PPC recovery % of being outside of 50 – 200% range. In most cases, dilution of the sample will reduce the concentration and activity of interfering substances. Samples should be diluted in LRW not exceeding the Maximum Valid Dilution which is calculated per USP (5) or USP (8).

Other interfering substances:

- **Some serine proteases (e.g. trypsin, activated blood factors) causing a false positive result must be denatured (for example, by heat treatment) before testing.**
- **Colored materials such as animal serum, albumin, and plasma**
- **Excessive turbidity**

If the procedure cannot be validated (1, 2) at a sample dilution that does not exceed MVD, the recombinant test cannot be used as an alternative test.

References

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4. Validation of Compendial Procedures <1225>, United States Pharmacopeia (current revision), United States Pharmacopeial Convention, Rockville, MD.
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7. Bacterial Endotoxins Test 4.01, Japanese Pharmacopoeia (current revision), Tokyo, Japan.
8. Medical Devices – Pyrogen and Endotoxins Testing <161>, United States Pharmacopeia (current revision), United States Pharmacopeial Convention, Rockville, MD.

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Application of a Recombinant Three-Factor Chromogenic Reagent for BET filed in the Pharmacopeias. Biol Pharm Bull (2019)42(12)2024-2037

Muroi M, Ogura N *et. al.*

Please contact Technical Services at techservice@acciusa.com if you have questions about using PyroSmart NextGen™.