Recombinant LAL Reagent

PyroSmart NextGen®

Instructions For Use

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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 (1,2,3). Guidance on validation of alternate test methods can be found in USP <1223> and <1225> (4,5) 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.

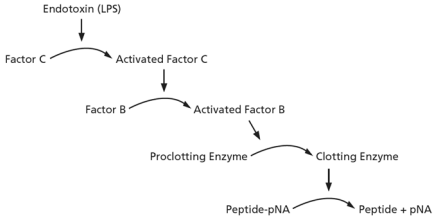


Figure 1: Cascade mechanism starting with endotoxin-activation of Factor C and yielding absorbance increase as a result of pNA release

Table 1: Materials Supplied with PyroSmart NextGen®
NOTE: Bulk packaging is available.

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

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 2: Storage conditions for PyroSmart NextGen®

Lyophilized Reagent	Store at 2-8°C. Before use, allow to equilibrate to room temp. for least 30 min
Reconstitution Buffer	Store at 2-8°C. Before use, allow to equilibrate to room temp. for least 30 min
Reconstituted Reagent	Room temp. Should be used within 3 to 20 minutes following reconstitution

1. PERFORMING PYROSMART NEXTGEN® IN AN ABSORBANCE MICROPLATE READER

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 the Vmean (Y-axis) against the standard concentration (X-axis) and is used to determine endotoxin concentrations in samples.

The software settings for both assay types are summarized in Table 3.

Table 3: Software Settings for PyroSmart NextGen® Assays in a Microplate Reader

	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 or Threshold mOD = 30	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 1. Additional materials and equipment that are required, but NOT supplied with PyroSmart NextGen® are listed in Table 4.

Table 4: Materials & Equipment Required but NOT Supplied with PyroSmart NextGen® for Microplate Reader Assays

Equipment Type	Specification	Description/Cat. #
Incubating absorbance microplate reader	Capable of maintaining a temperature of 37°C while collecting absorbance reads	e.g. BioTek® ELx808™, Molecular Devices readers or equivalent
Plate reader 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	e.g. ACC TB240-5, TB013-5, TB16C or equivalent
A set of adjustable single-channel micropipettes	Capable of delivering volumes of 5-20µL, 20-100µL and 100-1000 µL	Gilson, Rainin traditional or Eppendorf model fit the tips below or equivalent
Pipette tips	Free of interfering endotoxin Capable of delivering volumes of: 5-20µL, 20-100µL and 100-1000µL	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 a 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 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 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 it to equilibrate to 37°C.
2. Set up the software using appropriate settings (see Table 3).
3. Prepare the appropriate controls and samples
4. Prepare the test run as shown in Figure 2.
   The test setup is described in more detail below.
5. Read the test.

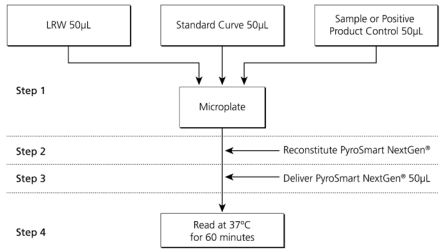


Figure 2: A Schema of the Test Procedure for Microplate Reader Assays

STEP 1: Transfer Test Sample

Transfer 50µL 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 the software plate layout.

STEP 2: Reconstitute PyroSmart NextGen® Recombinant LAL Reagent

Allow both the reagent and reconstitution buffer to equilibrate to room temperature. Gently tap the reagent 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. Gently swirl the vial for the 1st minute, then leave undisturbed for the next 2 minutes (a total of 3 minutes reconstitution before use). Do not mix again before use to avoid excessive foaming and a loss of sensitivity. If excessive mixing is utilized, leave undisturbed for 10 minutes or use a manual pipette to dispense to avoid bubbles. The reagent must be used within 20 min after reconstitution.

STEP 3: Deliver PyroSmart NextGen® to the microplate

Remove the plate cover. Fill a sterile combi-tip with the reconstituted reagent and set to deliver 50µL 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. With an electronic pipette, a dispensing speed of 5 or lower is recommended to avoid bubbles. 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.

2. PERFORMING PYROSMART NEXTGEN® IN PYROS® KINETIX FLEX TUBE READER

ASSAY CONDITIONS

PyroSmart NextGen® can be used to quantify endotoxin concentration as an *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.

The settings for tube reader software are described in Table 6 or Figure 3 depending on the type of software used.

Table 6: Pyros® EQS Software Settings for PyroSmart NextGen® Assays

	General Settings
Read	Kinetic, Absorbance
Wavelength	405nm
Reading Interval	10 sec
Runtime	80 min
Data Reduction	Threshold mOD = 20
Read	Kinetic, Absorbance
Baseline Adjustment	On, 125 – 325 seconds

Specific settings for Pyros® eXpress:

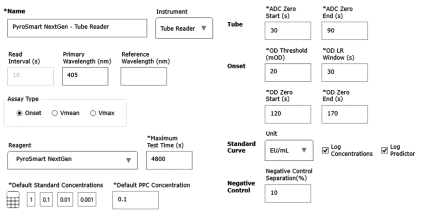


Figure 3: Pyros® eXpress Software Settings for PyroSmart NextGen® Assays

MATERIALS AND EQUIPMENT

Materials supplied with PyroSmart NextGen® are listed in Table 1. Additional materials and equipment that are required, but NOT supplied with PyroSmart NextGen® are listed in Table 7.

Table 7: Materials & Equipment Required but NOT Supplied with PyroSmart NextGen® for Tube Reader Assays

Equipment Type	Specification	Description/Cat. #
Incubating tube reader	Capable of maintaining a temperature of 37°C while collecting absorbance reads	Pyros® Kinetix Flex
Tube reader software	Allows for data reduction by Onset Time	Pyros® eXpress or Pyros® EQS
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 endotoxin	e.g. ACC WP050C or equivalent
8x75mm Depyrogenated Reaction Tubes	Borosilicate, free of interfering endotoxin	e.g. ACC TK100-10 or equivalent
Depyrogenated Glass Dilution Tubes	Free of interfering endotoxin	e.g. ACC TB240-5, TB013-5, TB16C or equivalent
A set of adjustable single-channel micropipettes	Capable of delivering volumes of 5-20µL, 20µL-100µL and 100-1000µL	Gilson, Rainin traditional or Eppendorf model fit the tips below or equivalent
Pipette tips	Free of interfering endotoxin, Capable of delivering volumes of: 5-20µL, 20-100µL and 100-1000µL	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 quivalent
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
Tube racks for reaction tubes	Provided with PK Flex	

+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 an example, refer to Table 8.

Table 8: Examples of Standard Curve Range and Setup

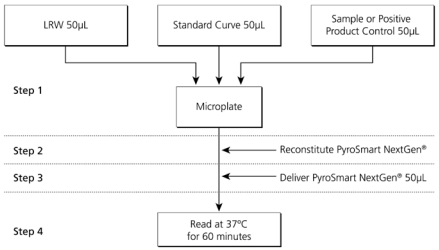
CSE (or RSE) concentration in EU/mL	Volume of LRW	CSE (or RSE) solution in EU/mL
50	-	-
1	4,900µL	100µL of 50 EU/mL
0.1	900µL	100µL of 1 EU/mL
0.01	900µL	100µL of 0.1 EU/mL
0.001	900µL	100µL of 0.01 EU/mL

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 1 to 0.001 EU/mL, spike 200µL of sample with 20µL of 1 EU/mL to render a final concentration of 0.1 EU/mL.

TEST PROCEDURE

1. Turn on the tube reader to allow it to equilibrate to 37°C.
2. Set up the software using appropriate settings (see Table 6 for Pyros® EQS or Figure 3 for Pyros® eXpress) for the specific software in use.
3. Prepare the appropriate controls and samples.
4. Prepare the test run as shown in Figure 4.
   The test setup is described in more detail below.
5. Read the test.

Figure 4: A Schema of the Test Procedure for Tube Reader Assays



STEP 1: Transfer Test Sample

Transfer 200µL of test sample (negative control x2, endotoxin standard series x2, sample dilutions x2 and PPC for each sample dilution x2) to the appropriate reaction tubes as defined in software layout.

STEP 2: Reconstitute PyroSmart NextGen® Recombinant LAL Reagent

Allow both the reagent and reconstitution buffer to equilibrate to room temperature. Gently tap the reagent vial to cause loose material to fall to the bottom. Break the vacuum by aseptically lifting the stopper. Discard the stopper. Transfer 2.8mL of PyroSmart NextGen® Reconstitution Buffer to the reagent vial and cover with Parafilm. Gently swirl the vial for the 1st minute, then leave undisturbed for the next 2 minutes (a total of 3 minutes reconstitution before use). Do not mix again before use to avoid excessive foaming and a loss of sensitivity. If excessive mixing is utilized, leave undisturbed for 10 minutes or use a manual pipette to dispense to avoid bubbles. The reagent must be used within 20 min after reconstitution.

STEP 3: Deliver PyroSmart NextGen® to the reaction tubes

Fill a sterile combi-tip with the reconstituted reagent and set to deliver 50µL aliquots, one aliquot at a time. With the pipette positioned at 45-degree angle relative to the reaction tube (while not touching the inside walls of the tube), deliver 50µL of the reagent to the first replicate of the negative control. Vortex mix the tube for 1 second and immediately insert into well no. 1 in the tube reader. Repeat for the remaining tubes of the negative controls. Then continue with the standard curve: lowest to the highest concentration one tube at a time. Then continue to samples.

STEP 4: Read the test

The test is started automatically with the insertion of each tube. Allow the test to run to completion.

ASSAY RUN VALIDITY CRITERIA FOR ALL ASSAYS

For the run to be valid, the conditions listed in Table 9 must be met.

Table 9: Examples of Standard Curve Ranges and Setups for All 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 (applies to plate reader method only):* 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 of endotoxin concentrations

Endotoxin concentrations of all test samples (including standards and controls) are calculated by interpolation against the standard curve using the equation for a straight line as follows:

- *Onset Time Assay:*
  Log Endotoxin Concentration = (Log Onset Time – Y-intercept) / Slope
- *Rate Assay (applies to plate reader method only):*
  Endotoxin Concentration = (Vmean – Y-intercept) / Slope

Polynomial regression can be also used for Onset Time assays.

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 PROCEDURES

The procedures are 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 pharmacopeial requirements (6,7,8 or 9).

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, 3) at a sample dilution that does not exceed MVD, the recombinant test cannot be used as an alternative test.

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