NCL Method STE-1
Version 1.0

Detection of Endotoxin Contamination by End Point Chromogenic LAL Assay

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

Method is written by: _____ 9 Mar 06.

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Testing facility: NCL, NCI-Frederick, Bldg 469, Room 250.

1. Introduction

This document describes a protocol for a quantitative detection of Gram negative bacterial endotoxin in nanoparticle preparations using an end-point Limulus Amebocyte Lysate (LAL) assay. The protocol is based on QCL-1000 kit insert manufactured by BioWhittaker/Cambrex Corporation (10.1) and the US FDA Guideline "Validation of the LAL test as an end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices" (10.2). Gram negative bacterial endotoxin catalyzes the activation of proenzyme in the Limulus Amebocyte Lysate. The activated enzyme then catalyzes the splitting of p-nitroanilin from the colorless substrate. The released p-nitroanilin is measured photometrically at 405 nm after reaction is terminated with a stop reagent. Concentration of endotoxin in a sample is in direct proportion with absorbance and is calculated from a standard curve. The assay requires approximately 1.0 mg of test nanomaterial.

2. Reagents

2.1. Sodium Hydroxide, Sigma, cat. #S2770

2.2. Hydrochloric acid, Sigma H9892

2.3. QCL-1000 LAL test kit, BioWhittaker

2.4. Glacial acetic acid, Sigma, cat#A9967

2.5. SDS, Sigma cat #L6026

2.6 Test-nanimaterial

Note: Equivalent reagents from other vendors can be used

3. Equipment

3.1. Pipettors covering range from 0.05 to 1 mL

3.2. Microcentrifuge tubes 1.5 mL

3.3. Microcentrifuge

3.4. Refrigerator, 2-8°C

3.5. Freezer, -20°C

3.6. Vortex

3.7. Pipet tips 0.5 μL – 1.0 mL

3.8. Plate reader

3.9. Disposable endotoxin-free glass dilution tubes (13x100mm)

3.10. Reagent reservoirs

3.11. Multichannel pipettor

3.12. Sterile 96-well plates

4. Reagent Preparation

4.1. Sodium Hydroxide

Prepare from concentrated stock by dilution into pyrogen-free LAL reagent water to make solution with final concentration of 0.1N.

4.2. Hydrochloric Acid

Prepare from concentrated stock by dilution into pyrogen-free LAL reagent water to make solution with final concentration of 0.1N.

5. Preparation of Standard Curve and Quality Controls

5.1. Preparation of Stock Solution

E.coli LPS supplied with the kit is a USP certified reference standard endotoxin (RSE) provided as a lyophilized powder. The contents of the vial containing RSE should be reconstituted with 1.0 mL of pyrogen-free LAL reagent water to a final concentration of 15-40 EU/mL. The actual concentration of the vial will be determined by value stated on the enclosed certificate of quality supplied with each kit. During reconstitution and prior to use, the stock solution should be vortexed vigorously and allowed to equilibrate to room temperature.

5.2 Preparation of stop solution

Prepare 25% v/v glacial acetic acid or 10% SDS in water for use as a stop solution.

5.3. Preparation of Calibration Standards

Level	Nominal Conc.,EU/mL	Preparation Procedure
Cal 1	1.0	100 μL stock + (X-1)/10 mL LAL reagent water*
Cal 2	0.50	500 μL Cal1 + 500 μL LAL reagent water
Cal 3	0.25	500 μL Cal2 + 500 μL LAL reagent water
Cal 4	0.10	100 μL Cal1 + 900 μL LAL reagent water

***** - X is concentration of the stock, e.g. if stock concentration is 23 EU/mL, then 100μL of this stock should be diluted with 2.2 mL [(23-1)/10] of LAL reagent water.

5.4. Preparation of Quality Controls

Level	Nominal Conc.,EU/mL	Preparation Procedure
QC1	0.4	100 μL Cal1+ 150 μL LAL reagent water

Legend: * - intermediate solution A is prepared only to make QC2 and is not used in assay.

5.5. Preparation of Inhibition/Enhancement Control

Level	Nominal Conc.,EU/mL	Preparation Procedure
*IntA**	*1.0*	10 μL stock + (X-1)/100 mL *NP suspension/solution***
IEC	0.4	100 μL IntA + 150 μL NP suspension/solution**

Legend: * - intermediate solution A is prepared only to make IEC and is not used in the assay; X is concentration of the stock, e.g. if stock concentration is 23 EU/mL, then 10μL of this stock should be diluted with 0.22 mL [(23-1)/100] of NP solution/suspension.** - concentration of nanoparticles should be equal to one assayed in test-sample.

6. Preparation of Study Samples

Study samples should be reconstituted in either LAL reagent water or sterile pyrogen-free PBS to a final concentration of 1.0 mg/mL. The pH of the study sample is checked using pH microelectrode and adjusted if necessary to be within the range 6.0-8.0 with sterile NaOH or HCl. Do not adjust pH of unbuffered solutions. To avoid sample contamination from microelectrode, always collect a small aliquot of the sample and use it to measure the pH. If sample was prepared in PBS, blank PBS should be tested in the assay.

7. Experimental Procedure

7.1. Prepare standard curve, quality control, inhibition/enhancement control and unknown samples as described above.

7.2. Create run template on a plate reader.

7.3. Carefully dispense 50 µL of LAL reagent water blank (4 wells), calibration standards (2 wells/each), controls (2 wells/each) and unknown samples (2 wells/each) into appropriate wells of pre-warmed to 37±1°C microplate.

7.4. Using multichannel pipet add 50µL of LAL reagent to all wells containing blanks, calibration standards, controls and unknown samples. Incubate for 10 minutes at 37±1°C.

7.5. Add 100 µL of pre-warmed substrate solution and incubate at a nominal temperature of 37±1°C for another 6 minutes.

7.6. Using multichannel pipet, add 100 µL of the stop solution to samples in the microplate and read absorbance at 405 nm.

8. Assay Acceptance Criteria

8.1. Linear regression algorithm is used to build standard curve. Precision (%CV) and accuracy (PDFT) of each calibration standard and quality control should be within 25%.

8.2. At least three calibration standards should be available in order for assay to be considered acceptable.

8.3. The correlation coefficient of the standard curve must be at least 0.980.

8.4. If quality controls fail to meet acceptance criterion described in 8.1 run should be repeated.

8.5. If standard curve fails to meet acceptance criterion described in 8.1-8.3 the run should be repeated.

8.6. Precision of the study sample should be within 25%.

8.7. Precision and accuracy of inhibition/enhancement control should be within 25% if no endotoxin is detected in a study sample and within 50% is endotoxin is detected in a study sample. If accuracy of IEC is outside of the specified above range, then study sample is interfering with the assay.

8.8. If sample interference is detected then analysis of diluted sample should be performed. Dilution of the study sample should not exceed minimum valid dilution (MVD). For detailed guide on how to prepare MVD please refer to reference 10.2. If no data such as maximum human (rabbit) dose or acceptable

limit are available for a given particle then MVD is calculated according to the following formula:

$$MVD = \frac{0.5\ (0.06)\ EU/mL \times 1.0\ mg/mL}{0.1\ EU/mL}$$

9. Sample Acceptance Criteria

9.1. The following limits are approved by the US FDA

Devices: 0.5EU/mL, except device in contact with CSF, for which limit is 0.06EU/mL.

Parenteral drugs: limit is equal to K/M, i.e. 5.0 EU/Kg /Maximum human (rabbit) dose/Kg administered in a single 1hour period.

Parenteral Drugs administered intrathecally: limit is equal to K/M, i.e. 0.2 EU/Kg /Maximum human dose/Kg administered in a single 1hour period.

9.2. The nanoparticle formulations will be treated as devices for acceptance/rejection, unless data for K/M formula are available.

10. References

10.1. Kinetic-QCL kit insert, BioWhittaker/Cambrex Corporation

10.2. FDA 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. December 1987.

10.3. Second Supplement to US 24-NF19. <85>. Bacterial Endotoxins. Rockville, MD: United States Pharmacopeia, 2000, 2875-2879.