



NCL Method STE-1.2

Detection and Quantification of Gram Negative Bacterial Endotoxin Contamination in Nanoparticle Formulations by Kinetic Turbidity 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.

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1. Introduction

This document describes a protocol for a quantitative detection of Gram negative bacterial endotoxin in nanoparticle preparations using a kinetic turbidity Limulus Amebocyte Lysate (LAL) assay. The protocol is based on the USP standard 85 “Bacterial endotoxin test” (1).

2. Principles

Gram negative bacterial endotoxin catalyzes the activation of proenzyme in the Limulus Amebocyte Lysate. In the presence of endotoxin LAL becomes turbid. The time taken to reach a particular level of turbidity (the onset time) is determined. Higher endotoxin concentrations give shorter onset times. Concentration of endotoxin in a sample is in direct proportion with onset time and is calculated from a standard curve prepared by spiking known concentration of endotoxin standard into LAL grade water. The assay requires approximately 1.0 mg of test nanomaterials and a special instrumentation to detect the turbidity. This method relies on PyrosKinetix Instrument.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

3.1 Reagents

- 3.1.1 Test nanomaterial
- 3.1.2 Sodium Hydroxide (NaOH) (Sigma, S2770)
- 3.1.3 Hydrochloric acid (HCl) (Sigma, H9892)
- 3.1.4 Control Endotoxin Standard, (Associates of Cape Code (ACC), E0005)
- 3.1.5 LAL Reagent (ACC, T0051)
- 3.1.6 LAL grade water (ACC, WP0501)

3.2 Materials

- 3.2.1 Pipettes and tips covering the range from 0.05 to 10 mL
- 3.2.2 Microcentrifuge tubes, 1.5 mL
- 3.2.3 Disposable endotoxin-free glass dilution tubes, 12x75 mm (ACC, TB240)

- 3.2.4 Disposable endotoxin-free reaction tubes, 8x75 mm (ACC, TK100)
- 3.3 Equipment
 - 3.3.1 Microcentrifuge
 - 3.3.2 Refrigerator, 2-8°C
 - 3.3.3 Freezer, -20°C
 - 3.3.4 Vortex
 - 3.3.5 Pyros Kinetix Instrument, ACC

4. Reagent Preparation

4.1 Sodium Hydroxide

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

4.2 Hydrochloric Acid

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

5. Preparation of Standard Curve and Quality Controls

5.1 Preparation of Stock Solution

E.coli lipopolysaccharide (LPS) supplied by ACC is a USP certified control standard endotoxin (CSE) provided as a lyophilized powder. The contents of the vial containing CSE should be reconstituted with ~3.5 - 5.0 mL of pyrogen-free LAL reagent water, depending on the potency. The final concentration this stock solution should be calculated for each lot and depends on product potency and amount supplied in each vial. The information about product potency and amount per vial can be found on the enclosed certificate of analysis supplied with each endotoxin standard. During reconstitution and prior to use, the stock solution should be vortexed vigorously for 30-60 sec, with 5-10 min settling times, over a 30-60 min time frame, and allowed to equilibrate to room temperature prior to use. Reconstituted endotoxin standard is stable for 4 weeks when stored at 2-8°C.

5.2 Preparation of LAL Reagent

LAL reagent is provided as lyophilized powder. Contents of each vial should be reconstituted per manufacturer's recommendations. Most vials will require reconstitution to a final volume of 5 mL.

5.3 Preparation of Calibration Standards

Sample	Nominal Concentration (EU/mL)	Preparation Procedure
Int. A	100*	100 µL Stock + 900 µL LAL reagent water
Int. B	10	100 µL Int. A + 900 µL LAL reagent water
Cal. 1	1.0	100 µL Int. B + 900 µL LAL reagent water
Cal. 2	0.1	100 µL Cal. 1 + 900 µL LAL reagent water
Cal. 3	0.01	100 µL Cal. 2 + 900 µL LAL reagent water
Cal. 4	0.001	100 µL Cal. 3 + 900 µL LAL reagent water

* This is an example; dilution of the CSE to make Cal. 1 and Int. solutions depends on the concentration of CSE stock and is determined for each lot of CSE reagent. Numbers shown in the table above are calculated based on stock concentration of 1000 EU/mL.

5.4 Preparation of Quality Controls

Sample	Nominal Concentration (EU/mL)	Preparation Procedure
Int. A*	100**	100 µL Stock + 900 µL LAL reagent water
Int. B*	10**	100 µL Int. A + 900 µL LAL reagent water
Int. C*	1.0**	100 µL Int. B + 900 µL LAL reagent water
QC1	0.05	50µL IntC +950 µL LAL reagent water

* Although concentrations of these solutions matche ones for Int. A, B and Cal 1 in Section 5.3, it is not a good practice to prepare calibrators and quality controls from the same intermediates.

** Intermediate solutions A, B, C are prepared only to make QC1 and are not used in assay.

5.5 Preparation of Inhibition/Enhancement Control

Sample	Nominal Concentration (EU/mL)	Preparation Procedure
Int. A*	100**	100 µL Stock + 900 µL LAL reagent water
Int. B*	10**	100 µL Int. A + 900 µL LAL reagent water
Int.C*	1.0**	100 µL Int. B + 900 µL LAL reagent water
IEC	0.05	25µ Int. C + 475 µL nanoparticle solution/suspension***

* Although concentrations of these solutions matche ones for Int. A, B and Cal 1 in Section 5.3, it is not a good practice to prepare calibrators and quality controls from the same intermediates.

** Intermediate solutions A, B, C are prepared only to make IEC and are not used in assay.

*** The 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 assay requires 1 mL of test sample. The pH of the study sample should be checked using a pH microelectrode and adjusted, if necessary, within the range of 6.0-8.0 using either sterile NaOH or HCl. Do not adjust the pH of unbuffered solutions. To avoid sample contamination from microelectrode, always remove a small aliquot of the sample for use in measuring the pH. If the sample was prepared in PBS, blank PBS should also be tested in the assay.

7. Experimental Procedure

7.1 Start PyrosKinetix software and create a new experiment template. Make sure instrument-computer communication is not interrupted. Make sure negative control sample is entered through the negative control panel, not through the sample panel, otherwise software will not be able to generate report.

7.2 Add 100 µL of negative control (water), calibration standards, quality control, IEC and test nanoparticles into pre-labeled glass tubes. Prepare duplicate tubes for each sample.

- 7.3 Using a repeating pipette, add 100µL of LAL reagent to first test vial, vortex it briefly, and insert into test slot in the instrument carousel. Repeat this procedure for other samples, processing one sample at a time. Allow instrument to run each point for no less than 7200 sec to allow time for samples with low amounts of endotoxin to develop. If no detectable endotoxin is present in the sample, the software will mark this sample as “not detected by 7200s”.

Note: some lots of the lysate are less sensitive than others, if the sensitivity of the particular lot is low, the time may need to be adjusted to 9600s or longer in order to allow the lowest calibrator to develop.

8. Assay Acceptance Criteria

- 8.1 Linear regression algorithm is used to construct the standard curve. Precision (%CV) of each calibration standard and quality control should be within 50%.
- 8.2 At least three calibration standards should be available in order for the 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 50%.
- 8.7 IEC is considered to be free of interference if the measured concentration of endotoxin added to the nanoparticle solution is between 50 and 200% of the known amount of endotoxin concentration, after subtraction of any endotoxin detected in the solutions of nanoparticles without added endotoxin (2).
- 8.8 If sample interference is detected, then analysis of a diluted sample should be performed. Dilution of the study sample should not exceed the minimum valid dilution (MVD). For a detailed guide on how to prepare MVD please refer to reference (2). If no data such as a maximum human (rabbit) dose or acceptable limits are available for a given particle, then MVD is calculated according to the following formula:

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

$$\text{MVD}_{(\text{devices} + \text{CSF})} = \frac{0.06 \text{ EU/mL} \times 1.0 \text{ mg/mL}}{0.1 \text{ EU/mL}}$$

9. Sample Acceptance Criteria

- 9.1 The following limits are approved by the US FDA:
- Devices: 0.5 EU/mL, except a device in contact with CSF, for which the limit is 0.06 EU/mL.
- Parenteral Drugs: the limit is equal to K/M. K is the mandated limit, 5 EU/kg. M is the maximum dose/kg administered in a single hour.
- Parenteral Drugs administered intrathecally: the limit is equal to K/M. K is the mandated limit, 0.2 EU/kg. M is the maximum dose/kg administered in a single hour.
- 9.2. According to USP 2007 (2), for anticancer products administered on a per square meter of body surface, the formula is K/M, where K is 5 EU/kg and M is (maximum dose/m²/hour x 1.80 m²)/70 kg
- 9.3. To convert an animal or human dose from mg/kg to mg/m², the dose in mg/kg is multiplied by the conversion factor of 37, indicated as k_m (for mass constant). The k_m factor has units of kg/m²; it is equal to the body weight in kg divided by the surface area in m². Example 2 mg/kg x 37 = 74 mg/m² (Ref.2)
- 9.4 Nanoparticle formulations will be treated as devices for acceptance/rejection, unless data for the K/M formula are available.

10. References

1. USP 34-NF29. <85>. Bacterial Endotoxins. Rockville, MD: United States Pharmacopeia, 2011, Volume 1, 78-81.
2. FDA Guidance for Industry and Reviewers Estimating the Safe Starting Dose in Clinical Trials for Therapeutics in Adult Healthy Volunteers. December 2002.

11. Abbreviations

CSF	cerebrospinal fluid
CV	coefficient of variation
EU	endotoxin unit
FDA	Food and Drug Administration
HCl	hydrochloric acid
IEC	inhibition/enhancement control
LAL	Limulus Amebocyte Lysate
LPS	lipopolysaccharide
MVD	minimum valid dilution
NaOH	sodium hydroxide
PBS	phosphate buffered saline
USP	United State Pharmacopeia