NCL Method STE-1.1

Detection and Quantification of Gram Negative Bacterial Endotoxin Contamination in Nanoparticle Formulations Using the End Point Chromogenic LAL Assay

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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 the QCL-1000 kit manufactured by Lonza Corporation [1] and the USP standard 85 "Bacterial endotoxin test" [2].

2. Principles

Gram negative bacterial endotoxin reacts with an enzyme in the Limulus Amebocyte Lysate. Following this interaction, a substrate is added to the test reaction. In the presence of activated enzyme, the substrate is cleaved, forcing the release of p-nitroaniline and generating a yellow color. The release of p-nitroaniline is terminated by addition of glacial acetic acid or SDS, and the substrate is measured spectrophotometrically at 405 nm. The concentration of endotoxin in a sample is directly proportional to the absorbance and can thus be calculated from a standard curve.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

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 QCL-1000 LAL test kit (Lonza, 50-648U)
- 3.1.5 Glacial acetic acid (Sigma, A9967)
- 3.1.6 LAL-grade water (Associates of Cape Cod, WP0501)

3.2 Materials

3.2.1 Pipettes, 0.05 to 10 mL

3.2.2 Microcentrifuge tubes, 1.5 mL

3.2.3 Disposable endotoxin-free glass dilution tubes, 12x75 mm (Associates of Cape Cod, TB240)

3.2.4 Reagent reservoirs

3.2.5 Multichannel pipettor

3.2.6 Sterile 96-well plates

3.3 Equipment

3.3.1 Microcentrifuge

3.3.2 Refrigerator, 2-8°C

3.3.3 Freezer, -20°C

3.3.4 Plate reader

3.3.5 Vortex

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.

4.3 Reconstitution of QCL-1000 Kit Reagents

Please refer to the instructions provided with the kit for reagent reconstitution and storage.

5. Preparation of Standard Curve and Quality Controls

5.1 Endotoxin Standard Stock Solution

E.coli lipopolysaccharide (LPS) supplied with the kit is a USP certified control standard endotoxin (CSE) provided as a lyophilized powder. The contents of the vial containing CSE 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 the value stated on the enclosed certificate of analysis 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 Stop Solution

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

5.3 Calibration Standards

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

* Stock is the sample from section 5.1. X is concentration of the stock, which varies between lots. Example calculation: if stock concentration is 23 EU/mL, then 100 µL of this stock should be diluted with 2.2 mL [(23-1)/10] of LAL reagent water. This instruction is also provided with each kit.

5.4 Quality Controls

Sample	Nominal Concentration (EU/mL)	Preparation Procedure
Int A*	1.0	10 µL of stock + (X-1)/100 µL**
QC1	0.4	100 µL Int.A + 150 µL LAL reagent water

* Intermediate solution A is prepared only to make QC1 and is not used in assay.

** Stock is the sample from section 5.1; X is concentration of the stock which varies between lots. Example of the calculation: if stock concentration is 23 EU/mL, then 10 µL of this stock should be diluted with 0.22 mL [(23-1)/100] of LAL reagent water. This instruction is also provided with each kit.

5.5 Inhibition/Enhancement Control

Sample	Nominal Concentration (EU/mL)	Preparation Procedure
Int. A*	1.0	10 µL stock + (X-1)/100 µL NP suspension/solution**
IEC	0.4	100 µL Int. A + 150 µL NP suspension/solution**

* Intermediate solution A is prepared only to make IEC and is not used in the assay.

** Stock is sample from section 5.1; X is concentration of the stock, e.g., if stock concentration is 23 EU/mL, then 10 µL of this stock should be diluted with 0.22 mL [(23-1)/100] of nanoparticle solution/suspension. The final concentration of nanoparticles in this sample should be equal to those assayed in the test, i.e. you will need to prepare an IEC for each nanoparticle dilution assayed.

Note: The volumes presented in the table are provided as an example. Volumes can be adjusted as needed, as long as the particle concentration in the IEC matches that in the test sample, and the final concentration of CSE is 0.4 EU/mL.

6. Preparation of Study Samples

Study samples should be reconstituted in either LAL reagent water or sterile, pyrogen-free PBS. 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.

The concentration of nanomaterial is unique to each formulation. The goal of this test is to measure the endotoxin level per mg of the test formulation, which commonly refers to the active pharmaceutical ingredient (API), but may also be measured in mg of total formulation or total element (e.g. gold or silver). The sample should be tested from the stock using several dilutions that do not exceed the Maximum Valid Dilution (MVD).

To determine the MVD one needs to know three parameters: endotoxin limit (EL), sample concentration and assay sensitivity (λ). EL is calculated according to the following formula:

$$EL = K/M$$

where K is maximum endotoxin level allowed per dose (5 EU/kg for all routes of administration except for the intrathecal route, for which K is 0.2 EU/kg) and M is the maximum dose to be administered per kg of body weight per single hour [2]. Note, estimation of EL for nanomaterials used as radiopharmaceuticals or as medical devices will be different; please refer to USP BET 85 for details [2]. When the dose information for the test nanomaterial is available based on an animal model (e.g. in mouse), one may use it to convert into human equivalent dose (HED). To do so, the animal dose is divided by the conversion factor specific to each animal species, e.g. 12.3 for mouse. Please refer to the FDA guideline for other conversion ratios [3]. Dose for cancer therapeutics is often provided in mg/m^2 instead of mg/kg. To convert an animal or human dose from mg/m^2 to mg/kg the dose in mg/kg is divided by the conversion factor of 37, indicated as k_m (for mass constant). The k_m factor has units of kg/m^2; it is equal to the body weight in kg divided by the surface area in m^2. Example 74 mg/m^2 /37 = 2 mg/kg [3].

The MVD is determined according to the following formula:

$$MVD = (EL \times \text{sample concentration})/\lambda$$

For example, when nanoparticle sample concentration is 10 mg/mL and its maximum dose in mouse is 123 mg/kg, the HED is 123/12.3 = 10 mg/kg. The EL for all routes except intrathecal would therefore be 0.5 EU/mg (5 EU/kg/10mg/kg) and the MVD would be 50 ((0.5 EU/mg x 10 mg/mL)/0.1 EU/mL). In this case, the nanomaterial will be tested directly from stock and at several dilutions not exceeding 50, e.g., 5, 10 and 50. When the information about the dose is unknown, the highest final concentration of the test nanomaterial is 1 mg/mL and the MVD is 5. It is very important to recognize that if the dose, route of administration and/or the sample concentration for the test nanomaterial change, the EL and MVD will also change.

Important: Nanomaterials with an absorbance overlapping with 405 nm will interfere with this assay and therefore should be tested using other versions of the LAL (e.g. gel-clot and turbidity).

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 plate reader.

7.3 Carefully dispense 50 µL of LAL reagent water (4 wells), calibration standards (2 wells/each), controls (2 wells/each) and unknown samples (2 wells/each) into appropriate wells of a pre-warmed (37 ± 1°C) sterile 96-well microplate. See Appendix for an example plate map.

7.4 Using a multichannel pipet, add 50 µL 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 chromogenic substrate solution and incubate at a nominal temperature of 37 ± 1°C for another 6 minutes.

7.6 Using a multichannel pipet, add 100 µL of the stop solution (section 5.2) to samples in the microplate and read absorbance at 405 nm.

8. Assay Acceptance Criteria

8.1 Linear regression algorithm is used to construct the 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 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, the 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 of inhibition/enhancement controls should be within 25%

8.8 Spike recovery indicative of the accuracy of the inhibition/enhancement control should between 50 and 200% [2]. Spike recovery <50% is indicative of inhibition; that above 200% is indicative of either endotoxin contamination or enhancement.

8.9 If sample interference is detected, the results for this sample are invalid. Other tests should be considered as discussed in reference 4.

9. Sample Acceptance Criteria

9.1 Endotoxin levels in the sample are acceptable if within the EL calculated for the given formulation (please refer to section 6 and reference 4 for details).

10. References

1. Kinetic-QCL kit insert, BioWhittaker/Cambrex Corporation
2. USP 34-NF29. <85>. Bacterial Endotoxins. Rockville, MD: United States Pharmacopeia, 2011, Volume 1, 78-81.
3. FDA Guidance for Industry and Reviewers Estimating the Safe Starting Dose in Clinical Trials for Therapeutics in Adult Healthy Volunteers. December 2002.
4. US FDA. Guidance for Industry. Pyrogen and Endotoxins testing: Questions and answers, 2012

11. Abbreviations

API	active pharmaceutical ingredient
CSE	control standard endotoxin
CV	coefficient of variation
EU	endotoxin unit
EL	endotoxin limit
FDA	Food and Drug Administration
HCl	hydrochloric acid
HED	human equivalent dose
IEC	inhibition/enhancement control
LAL	Limulus Amebocyte Lysate
LPS	lipopolysaccharide
MVD	maximum valid dilution
NaOH	sodium hydroxide
PBS	phosphate buffered saline
PDFT	percent difference from theoretical
SDS	sodium dodecyl sulfate

USP	United State Pharmacopeia
v/v	volume/volume ratio

12. Appendix

Example Plate Map. Sterile plate should be pre-warmed to 37°C before adding samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	LAL water	Cal. 1	Cal. 2	Cal. 3	Cal. 4	QC 1	IEC 1	TS 1	IEC 2	TS 2	QC 1	LAL water
B	LAL water	Cal. 1	Cal. 2	Cal. 3	Cal. 4	QC 1	IEC 1	TS 1	IEC 2	TS 2	QC 1	LAL water
C												
D												
E												
F												
G												
H												

Cal, Calibration Standards; QC, Quality Control; IEC, Inhibition/Enhancement Control; TS, Test Sample