NCL Method STE-1.4

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

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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 chromogenic Limulus Amebocyte Lysate (LAL) assay. The protocol is based on USP standard 85, "Bacterial endotoxin test" [1].

2. Principles

Gram negative bacterial endotoxin catalyzes the activation of proenzyme in the Limulus Amebocyte Lysate. The enzyme, activated in the presence of endotoxin, cleaves a chromogenic substrate and the product of this reaction is detected by reading sample absorbance at 405 nm. Higher endotoxin concentrations give greater absorbance at 405 nm. The concentration of endotoxin in a sample is in direct proportion to the absorbance, and is calculated from a standard curve prepared by spiking known concentrations of endotoxin standard into LAL grade water. This method relies on the PyrosKinetixFlex instrument by Associates of Cape Cod Inc. (ACC). Data analysis is performed using PyrosEQS software (ACC).

3. Reagents 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

1. Control Endotoxin Standard (ACC, EC010)
   Note: this CSE is different than that used for turbidity and gel-clot LAL
2. Pyrochrome LAL Reagent (ACC, C1500-25)
3. LAL grade water (ACC, WP0501)
4. Sodium Hydroxide (NaOH) (Sigma, S2770)
5. Hydrochloric acid (HCl) (Sigma, H9892)
6. Test nanomaterial

3.2 Materials

1. Pyrogen-free pipettes and tips, 0.05 to 10 mL (RAININ)
2. Pyrogen-free microcentrifuge tubes, 1.5 mL

3. Repeat pipettor
4. Combitip, 5 mL pyrogen free
5. Disposable endotoxin-free glass dilution tubes, 12x75 mm (ACC, TB240)
6. Disposable endotoxin-free reaction tubes, 8x75 mm (ACC, TK100)

3.3 Equipment

1. Microcentrifuge
2. Refrigerator, 2-8°C
3. Freezer, -20°C
4. Vortex
5. Pyros Kinetix or PyrosFlex 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.

4.3 Control Standard Endotoxin (CSE)

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 approximately 1.8 - 5.0 mL of pyrogen-free LAL reagent water, depending on the potency. The final concentration of 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.

4.4 Preparation of LAL Reagent

LAL reagent is supplied as a lyophilized powder. The contents of each vial should be reconstituted per manufacturer's recommendations and are performed in Glucashield buffer, supplied along with the reagent. Most vials will require reconstitution to a final volume of 3.2 mL.

5. Preparation of Standard Curve and Quality Controls

5.1 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 provided only as an example. Dilution of the CSE to make Int. and Cal. solutions depends on the concentration of the CSE stock and is determined for each lot of CSE reagent. Numbers shown in the table above are calculated based on a stock concentration of 1000 EU/mL.

5.2 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	50 µL Int. C + 950 µL LAL reagent water

* Although concentrations of these solutions match those for Int. A, B and Cal. 1 in Section 5.1, it is not good practice to prepare calibrators and quality controls from the same intermediates.

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

5.3 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	25 µL Int. C + 475 µL nanoparticle solution/suspension***

* Although concentrations of these solutions match ones for Int. A, B and Cal 1 in Section 5.1, it is not 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 the assay.

*** The concentration of nanoparticles should be equal to one assayed in a test-sample. You will need to prepare IEC for each nanomaterial dilution assayed in this test.

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 is to measure 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 using several dilutions from the stock, not exceeding the Maximum Valid Dilution (MVD).

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

$$EL = K/M$$

where K is the 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 [1]. Note, estimation of EL for nanomaterials used as radiopharmaceuticals or as medical devices will be different; please refer to USP BET 85 for details [1]. When the dose information for the test nanomaterial is available based on an animal model (e.g. in mouse), it can be converted 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 [2]. 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 [2].

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. EL for all routes except

intrathecal would therefore be 0.5 EU/mg (5 EU/kg / 10 mg/kg). MVD would be 5000 [(0.5 EU/mg x 10 mg/mL) / 0.001 EU/mL]. In this case, the nanomaterial would be tested directly from the stock and at several dilutions not exceeding 5000, e.g. 5, 50, 500 and 5000. When information about the dose is unknown, the highest final concentration of test nanomaterial is 1 mg/mL and the MVD is 500. 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: Nanoformulations with absorbance overlapping at 405 nm will interfere with this assay and therefore should be tested using other versions of LAL (e.g. gel-clot and turbidity). Turbid formulations with low MVD will likely interfere with both turbidity and chromogenic LALs and should be tested using the gel-clot and other methods as discussed in reference 3.

7. Experimental Procedure

1. Turn the instrument on approximately 20-30 minutes before starting the assay to allow the instrument to warm up. Set up detection wavelength to 405 nm, which is the absorbance for the detection of cleaved chromogenic substrate.
2. Start the PyrosKinetix or PyrosFlex 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.
3. Add 200 µL of negative control (water), calibration standards, quality control, IEC and test nanoparticles into pre-labeled glass tubes. Prepare duplicate tubes for each sample.
4. Using a repeating pipette, add 50 µL 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”.
5. Note: some lots of the lysate are less sensitive than others, if the sensitivity of a particular lot is low, the time may need to be adjusted to 9600 sec or longer in order to allow the lowest calibrator to develop.

8. Assay Acceptance Criteria

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%.
2. At least three calibration standards should be available for assay to be considered acceptable.
3. The correlation coefficient of the standard curve must be at least 0.980.
4. If quality controls fail to meet acceptance criterion described in 8.1, run should be repeated.
5. If standard curve fails to meet acceptance criterion described in 8.1-8.3, the run should be repeated.
6. Precision of the study sample should be within 25%.
7. Precision of inhibition/enhancement control should be within 25%.
8. Spike recovery indicative of the accuracy of the inhibition/enhancement control should between 50 and 200% [1]. Spike recovery less than 50% is indicative of inhibition; that above 200% is indicative of either endotoxin contamination or enhancement.
9. If sample interference is detected, the assay results for this sample are invalid. Other tests should be considered as discussed in reference 3.

9. Sample Acceptance Criteria

Endotoxin level in the sample is acceptable if it is within the EL calculated for the given formulation (please refer to section 6 and reference 3 for details).

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.
3. US FDA. Guidance for Industry. Pyrogen and Endotoxins testing: Questions and answers, 2012.

11. Abbreviations

API	active pharmaceutical ingredient
BET	bacterial endotoxin test
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
USP	United State Pharmacopeia