NCL Method STE-1.3

Detection and Quantification of Gram Negative Bacterial Endotoxin Contamination in Nanoparticle Formulations by Gel-Clot 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 discusses the quantitative detection of Gram negative bacterial endotoxin in nanoparticle preparations using the gel-clot Limulus Amebocyte Lysate (LAL) assay. The protocol for this assay is based on instructions provided with the reagents from Associates of Cape Cod as well as USP standard 85, "Bacterial endotoxin test [1]. In lieu of detailing the exact procedure here (which can be found in reference [1], a "Bench Sheet" is provided that can be used in conjunction with the USP protocol.

2. Principles

Gram negative bacterial endotoxin reacts with an enzyme in the Limulus Amebocyte Lysate, resulting in activation of a proteolytic cascade leading to clotting of the lysate. The concentration of endotoxin in a sample is determined by sample titration to an endpoint.

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

1. Control Endotoxin Standard (ACC, E0005)
2. LAL Reagent (ACC, G5003)
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. Disposable endotoxin-free glass dilution tubes, 12x75 mm (ACC, TB240)
4. Gel-clot test tubes (ACC, TS050)
5. Tube racks

3.3 Equipment

1. Microcentrifuge
2. Refrigerator, 2-8°C
3. Freezer, -20°C
4. Water bath (**Important**: Do NOT use circulating bath.)

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 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 166.7 [(0.5 EU/mg x 10 mg/mL) / 0.03 EU/mL]. In this case, the nanomaterial would be tested directly from the stock and at several dilutions not exceeding 166.7, e.g. 5, 75 and 150 (or 166). When information about the dose is unknown, the highest final concentration of test nanomaterial is 1 mg/mL and the MVD is 16.7. 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.

6. Procedure

6.1 Overview

The gel clot LAL procedure overviewed here follows the USP BET 85 protocol. For complete details on this procedure, please consult the USP reference [1]. Outlined below is a "Bench Sheet" that can be printed and used alongside the USP protocol.

Briefly, the test includes three tests:

Test 1: Confirmation of labeled lysate sensitivity

Test 2: Test for interfering factors

Test 3: Endotoxin assessment in the test sample by either limit test or quantitative test

Test 1 can be done once and need not be repeated until bacterial endotoxin standard and lysate lots have changed. Test 2 is conducted to identify any potential interference of the test sample with the LAL gel-clot procedure. The qualitative (limit test) or quantitative test of Test 3 is done only after absence of interference has been confirmed in Test 2.

The qualitative (limit) test results are negative when both replicates do not clot. If clotting was observed in one replicate, the test has to be repeated. If in the repeated test one or both replicates clot, the sample contains endotoxin contamination at a level equal to or greater than the assay sensitivity. If a diluted sample was tested, the assay sensitivity should be multiplied by the dilution factor to report the limit of endotoxin contamination in the sample.

The quantitative test determines endotoxin concentration in the sample as endpoint concentration of the replicates with a positive response (i.e., clotting). If none of the replicates of the valid assay give a positive response, the concentration of endotoxin is reported as below the lysate sensitivity. If all replicates are positive, then concentration of endotoxin is reported as greater than or equal to the greatest dilution multiplied by the assay sensitivity.

6.2 General Procedure

1. Label as many reaction tubes as needed to accommodate the number of test samples. Refer to bench sheet for details about number of replicates used in Test 1, 2, and 3 of the assay.
2. Aliquot 100 μL of water, controls or test sample per tube.
3. Prepare CSE such that the final concentration is equal to 4λ. When 100 μL of this standard is combined with 100 μL of water or test sample, the final concentration of CSE is equal to 2λ.
4. Add 100 μL of lysate per test tube, vortex briefly, then place entire rack into a 37°C water bath for 1 hr.
5. Remove samples from water bath and dry using paper towel.
6. Invert the tube using smooth motion and record results using “+” (firm clot) or “-“ (no clot or loose clot) on the bench sheet.
7. Proceed with analysis according to USP BET 85; use bench sheet as supporting material.

Test 1 – Qualification of Reagent Sensitivity
(Perform once with each new reagent lot)

1. Information About Test

Incubation Time:	**Start:**	**Finish:**
Temperature:	**Start:**	**Finish:**

Date _____ **Tested by** _____

2. Test Results

Record test results in the table below. If a firm gel has formed that remains in place under inversion, the result is "+" (or positive). If an intact gel is not formed the result is "-" (or negative).

Replicate Number	**Endotoxin Standard Concentration, EU/mL**				
	2λ	**1λ**	**0.5λ**	**0.25λ**	**Water**
1					
2					
3					
4					

The test is valid if the lowest concentration of the tested standard solution is negative in all replicates. Please check here to confirm this is the case_____.

3. Calculation of Geometric Mean Sensitivity (Reagent Qualification)

The endpoint is the smallest concentration in the series of decreasing concentrations of CSE that clots the lysate.

Geometric Mean Endpoint Concentration = Antilog ($\sum e/f$),

where $\sum e$ is the sum of the log end-point concentrations of the dilution series used, and f is the number of replicate test tubes. The Geometric Mean Endpoint Concentration = λ or assay sensitivity. Please enter λ value calculated in this section into the far right column of the table in section 4 below

4. Reagent Qualification Summary

Reagents	**Lot #**	**Expiration**	**Sensitivity, EU/mL**
Pyrotell			
Endotoxin Standard			**N/A**
Water			**N/A**

Test 2 – Inhibition Enhancement Control

Important Note: This test should be repeated for each nanoparticle concentration. Ideally, undiluted sample is tested first. If interference is found for undiluted sample, repeat test 2 with as many dilutions as necessary to overcome the interference, ensuring the dilutions do not exceed the MVD.

1. Information About Test

Incubation Time:	**Start at:**	**Finish at:**
Temperature:	**Start at:**	**Finish at:**

Date _____ **Tested by** _____

2. Test Samples

Please enter EL_____EU/mg and MVD_____

*Nanoparticles are from stock_____or at initial dilution_____or MVD_____

Nanoparticle stock concentration_____mg/mL by API_____total_____other_____

Concentration is based on client's provided data_____or NCL measured data_____

To prepare samples B1 and C1, spike CSE at a final concentration 2λ into nanoparticles and LAL water, respectively. Next, perform three serial 1:2 dilutions of B1 and C1 in nanoparticle solution (samples B2-B4) and water (samples C2-C4), respectively. Refer to the table below for information about sample name, dilution factor, number of replicates and endotoxin concentration. A replicate here refers to one test tube.

Sample	Sample Description	Number of Replicates	Dilution Factor	Endotoxin Concentration
A	**Nanoparticle solution***	**4**	**none**	**-**
B1	**CSE in nanoparticle solution***	**4**	**1**	**2 λ**
B2	**CSE in nanoparticle solution***	**4**	**2**	**1 λ**
B3	**CSE in nanoparticle solution***	**4**	**4**	**0.5 λ**
B4	**CSE in nanoparticle solution***	**4**	**8**	**0.25 λ**
C1	**CSE in LAL water**	**2**	**1**	**2 λ**
C2	**CSE in LAL water**	**2**	**2**	**1 λ**
C3	**CSE in LAL water**	**2**	**4**	**0.5 λ**
C4	**CSE in LAL water**	**2**	**8**	**0.25 λ**
D	**LAL water**	**2**	**none**	**-**

(Test 2 – Inhibition Enhancement Control continues on the next page.)

3. Record Test Results in the Table Below

Sample	Replicate 1	Replicate 2	Replicate 3	Replicate 4
A				
B1				
B2				
B3				
B4				
C1				
C2				
C3				
C4				
D				

4. Analysis and Interpretation

Calculate geometric mean sensitivity of sample B and C using the formula described in Test 1, Section 3. Record the data below:

Sample B: _____ EU/mL
Sample C: _____ EU/mL

Using data from the above table (Test 2, Section 3) and calculation of geometric mean sensitivity, confirm the following points:

The test result of sample A is negative_____
The test result of sample D is negative_____
The test result of sample C confirms the assay sensitivity_____

- *If the answer to all these points is yes,* ***the test is valid*** _____*(please check to confirm)*
- *If A is positive, the nanoparticle test sample interferes with the assay and* ***the test is invalid*** _____*(please check to confirm)*
- The sensitivity of the lysate determined in the presence of nanoparticles (sample B) is not less than 0.5λ and not more than 2λ_____
    - *If the answer to this point is yes, the nanoparticle test sample at the tested concentration* ***does not contain*** *substances interfereing with the gel-clot LAL*_____*(please check to confirm)*
    - *If the answer to this point is no, the nanoparticle* ***test sample interferes*** *with LAL*_____*(please check to confirm)*

If the test is valid proceed to Test 3A or 3B. Choice between 3A and 3B depends on the project need.

Test 3A – Limit Test

Important Note: This test is done at the highest nanoparticle concentration (lowest dilution of the stock nanoparticle sample) not interfering with gel-clot LAL. Refer to Test 2 for information about this concentration.

1. Information About Test

Incubation Time:	**Start:**	**Finish:**
Temperature:	**Start:**	**Finish:**

Date _____ **Tested by** _____

2. Record Test Results in the Table Below

*Nanoparticles are from stock_____or at initial dilution_____or MVD_____

Nanoparticle stock concentration_____mg/mL by API_____total_____other_____

Concentration is based on client's provided data_____or NCL measured data_____

Sample	Sample Description	Dilution Factor	Endotoxin Conc.	Replicate 1	Replicate 2
A	**Nanoparticles***		**-**		
B	**2 λ in Nanoparticles***		**2 λ**		
C	**2 λ LAL water**		**2 λ**		
D	**LAL water**		**-**		

3. Analysis and Interpretation

Using data from the above table (Test 3A, Section 2) confirm the following points:

Both replicates of sample B are positive_____
Both replicates of sample C are positive _____
Both replicates of sample D are negative _____

If the answer to all these points is yes, ***the test is valid***_____*(please check to confirm)*

- Both replicates of sample A are negative_____= **nanoparticle complies with the test**
- Both replicates of sample A are positive _____= **nanoparticle does not comply with the test**
- One replicate of sample A is positive_____= repeat test one more time
    - Both replicates of sample A in repeat test are negative_____= **nanoparticle complies with the test**
    - One or both replicates of sample A in repeat test is positive_____= **nanoparticle does not comply with the test**

Test 3B – Quantitative Test

Important Note: This test is done at the highest nanoparticle concentration (lowest dilution of the stock nanoparticle sample) not interfering with gel-clot LAL. This dilution is called "initial dilution". Refer to Test 2 for information about this concentration.

1. Information about Test

Incubation Time:	**Start:**	**Finish:**
Temperature:	**Start:**	**Finish:**

Date _____ **Tested by** _____

2. Record Test Results in the Table Below

Sample	Sample Description	Dilution Factor	Endotoxin Conc.	Replicate 1	Replicate 2
A1	**Nanoparticles***	**1**	**-**		
A2	**Nanoparticles***	**2**	**-**		
A3	**Nanoparticles***	**4**	**-**		
A4	**Nanoparticles***	**8**	**-**		
B	**Nanoparticles* +2λ Endotoxin Std**	**1**	**2λ**		
C1	**Water+ 2λ Endotoxin Std**	**1**	**2λ**		
C2	**Water+ 1λ Endotoxin Std**	**2**	**1λ**		
C3	**Water+ 0.5λ Endotoxin Std**	**4**	**0.5λ**		
C4	**Water+ 0.25λ Endotoxin Std**	**8**	**0.25λ**		
D	**Water**	**-**	**-**		

* The concentration of nanoparticles in this sample is the one selected in Test 2, for purposes of this test it is called "initial dilution". Subsequent dilutions of the initial dilution should be done in a way such that the final dilution does not exceed the MVD. For example if the MVD is 166.7 and the initial dilution of nanoparticles to a concentration not interfering with the LAL is 20, analysis of this sample at dilutions shown in the dilution factor column are within the MVD (20x8 = 160, i.e. <166.7). Likewise, if the initial dilution is 40, then subsequent dilution 8 will be above the MVD (40x8 = 320, i.e. >166.7).

3. Result Evaluation

3.1 Calculate geometric mean end point concentration for sample C according to formula described in Test 1, Section 3. Use the table below to record observations.

3.2 Test is valid if all of the following conditions are met:

Condition	Yes (+)
Both replicates of sample D are negative	
Both replicates of sample B are positive	
The geometric mean endpoint concentration of sample C is between 2λ and 0.5λ	

(Test 3 – Quantitative Test continues on the next page.)

3.3 Calculate endotoxin concentration in nanoparticle sample (Sample A):

1. Calculate the endpoint concentration for each replicate by multiplying each endpoint dilution factor by λ. Record results in the table below.

Dilution Factor	Endpoint Concentration, EU/mL
1	**λ x 1 =**
2	**λ x 2 =**
4	**λ x 4 =**
8	**λ x 8 =**

2. Consider the following points:

   - The endotoxin concentration of nanoparticle solution is the endpoint concentration of the replicates. The endpoint concentration is the lowest concentration in the series of decreasing concentrations of CSE that clots the lysate.
   - If the test is conducted with diluted sample, the endotoxin concentration in the stock nanoparticle is the endpoint concentration multiplied by the dilution factor used to prepare the intermediate dilution analyzed in the assay.
   - Record endpoint concentration here_____ x dilution factor = _____EU/mg.
   - If none of the dilutions of the test sample are positive in a valid assay, report the endotoxin concentration as < λ_____.
   - If diluted sample was analyzed, report concentration as < λ x lowest dilution.
   - If all dilutions are positive, the endotoxin concentration is ≥ λ x initial dilution factor x _____.

7. 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.

8. Abbreviations

API	active pharmaceutical ingredient
BET	bacterial endotoxin test
CSE	control standard endotoxin
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
MVD	maximum valid dilution
NaOH	sodium hydroxide
PBS	phosphate buffered saline
PCC	physicochemical characterization
USP	United State Pharmacopeia