NCL Method STE-1.3

Detection and Quantification of Gram Negative Bacterial Endotoxin Contamination in Nanoparticle Formulations by Gel-Clot LAL Assay

Nanotechnology Characterization Laboratory
National Cancer Institute-Frederick
SAIC-Frederick
Frederick, MD 21702
(301) 846-6939
ncl@mail.nih.gov

revised, September 2011

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 written by:

Marina A. Dobrovolskaia, Ph.D. (NCL)

Barry Neun, B.S. (NCL)

1. Introduction

This document discusses the quantitative detection of Gram negative bacterial endotoxin in nanoparticle preparations using the gel-clot Limulus Amebocyte Lysate (LAL). The protocol for this assay is based on instructions provided with the reagents from Associates of Cape Code as well as the 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 which results 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. The assay requires approximately 1.0 mg of test nanomaterial.

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, G5003)
- 3.1.6 LAL grade water (ACC, WP0501)

3.2 Materials

- 3.2.1 Pipettes 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, 13x100 mm (ACC, TB013)

3.2.4 Gel-clot test tubes (ACC, TS050)

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 Water bath

3.3.6 tube racks

4. Procedure

The gel clot LAL procedure indicated here follows the protocol written by the USP. 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 steps: Step 1, preparatory testing; Step 2, limit test; and Step 3, quantitative test. The preparatory test is done to confirm the labeled sensitivity of the lysate. This test can be done once and need not to be repeated until bacterial endotoxin standard and lysate lots have changed. The limit test is conducted to identify any potential interference of the test sample with the LAL gel-clot procedure. The quantitative test is done only after absence of interference has been confirmed in the limit test. The quantitative test determines endotoxin concentration in the sample as endpoint concentration of the replicates with 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 that below the lysate sensitivity. If all replicates are positive then concentration of endotoxin is reported as greater than or equal to that of the greatest dilution multiplied by the assay sensitivity.

Step 1 - Qualification of Reagent Sensitivity
(Do once with each new reagent lot)

1. Information About Test

Incubation	Start	Finish
Time		
Temperature		

Date _____ **Tested by** _____

2. Test Results

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

3. Calculation of Geometric Mean Sensitivity (Reagent Qualification)

Antilog $\sum_{\text{log sensitivity}}$ / number of replicates = _____ **EU/mL**

4. Reagent Qualification Summary

Reagents	Lot #	Expiration	Sensitivity
Pyrotell			
Endotoxin Standard			N/A
Water			N/A

Step 2 – Inhibition Enhancement Control

1. Information About Test

Incubation	Start	Finish
Time		
Temperature		

Date _____ **Tested by** _____

2. Test Samples (Nanoparticles are at dilution equal to 8MVD)

Sample	Endotoxin Std Concentration	Number of Replicates	Fill in Actual Std. Conc., EU/mL
A	None in nanoparticle solution	4	0
B1	2λ in nanoparticle solution	4	
B2	1λ in nanoparticle solution	4	
B3	0.5λ in nanoparticle solution	4	
B4	0.25λ in nanoparticle solution	4	
C1	2λ in LAL water	2	
C2	1λ in LAL water	2	
C3	0.5λ in LAL water	2	
C4	0.25λ in LAL water	2	
D	None in LAL water	2	0

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				

Test is acceptable if A and D are negative; if A is positive – nanoparticles either have endotoxin contamination or interfere with the assay.

4. Calculation of Geometric Mean Sensitivity

Sample B: Antilog $\sum_{\text{log sensitivity}}$ / number of replicates = _____ EU/mL
Sample C: Antilog $\sum_{\text{log sensitivity}}$ / number of replicates = _____ EU/mL

Compare sensitivity for samples B and C. Nanoparticles do not interfere with assay if sensitivity in sample B is between 2λ and 0.5λ. If IEC is acceptable proceed to Step 3.

Step 3 – Quantification of Endotoxin in Nanoparticle Sample

1. Information About Test

Incubation	Start	Finish
Time		
Temperature		

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 endotoxinStd	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	-	-		

3. Result Evaluation
(NOTE: Geometric means need to be calculated only when replicates do not match.)

3.1 Calculate geometric mean end point concentration for sample C according to formula:

Antilog $\sum_{\text{log sensitivity}}$ / number of replicates = _____ EU/mL

3.2. Test is valid if the following conditions are met:

Condition	Yes (+) or No (-)
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	

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

Dilution Factor	Endpoint Concentration, EU/mL	Geometric Mean Endpoint Concentration = Endotoxin Concentration in Nanoparticle Sample
1	λ x 1 = _____	Antilog $\sum_{\text{log sensitivity}}$ / number of replicates = _____ EU/mL
2	λ x 2 = _____	
4	λ x 4 = _____	
8	λ x 8 = _____	

5. References

1. USP 34-NF29. <85>. Bacterial Endotoxins. Rockville, MD: United States Pharmacopeia, 2011,Volume 1, 78-81.

6. Abbreviations

EU	endotoxin unit
FDA	Food and Drug Administration
IEC	inhibition/enhancement control
LAL	Limulus Amebocyte Lysate
MVD	minimum valid dilution
USP	United State Pharmacopeia