Detection and Quantification of Gram Negative Bacterial Endotoxin Contamination in Nanoparticle Formulations by Gel-Clot 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 a kinetic turbidimetric 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 the USP standard 85 "Bacterial endotoxin test [5]. A "Bench Sheet" is provided that can be used in conjunction with the USP protocol.

2 Principle of the Method

This method relies on an *in vitro* end-product endotoxin test which utilizes a *Limulus Amebocyte* Lysate (LAL), an extract of blood cells (amebocytes) from the horseshoe crab. The method is designed to detect endotoxin activity by clotting of the lysate after incubating the reaction mixture at controlled temperature of 37°C.

Gram negative bacterial endotoxin catalyzes the activation of proenzyme in the Limulus Amebocyte Lysate. Bang[1] observed in 1956 that the infection of the horseshoe crab *Limulus polyphemus* with Gram-negative bacteria resulted in intravascular coagulation, as a result of a reaction between endotoxin and a clotting protein in amebocytes of *Limulus*[2]. A LAL proenzyme is activated in the presence of endotoxin. As a result of the following cascade of enzyme activation steps coagulation is initiated leading to clotting of the lysate. This method relies on Limulus Amebocyte Lysate PYROTELL® by Pyroquant Diagnostik GmbH, a subsidiary of Associates of Cape Cod, Inc. (ACC) [3]. The protocol for this assay is based on instructions provided with the reagents from Associates of Cape Cod.

3 Applicability and Limitations (Scope)

This SOP was developed to determine and quantify endotoxin contamination of different nanomaterials. This SOP was created according to DIN EN ISO 297014 adapted for the analysis nanomaterials[4] and according to the suppliers instruction for the use of the Kinetic Turbidimetric Microplate LAL Assay Pyrotell [3]. Use Pyrotell-T for in vitro diagnostic purposes only.

4 Related Documents

Table 1:

Document ID	Document Title
NCL Method STE-1.3	**Detection and Quantification of Gram Negative Bacterial Endotoxin Contamination in Nanoparticle Formulations by Gel-Clot LAL Assay**

5 Equipment and Reagents

5.1 Equipment

5.1.1 Pyrogen-free microcentrifuge tubes, 1.5 mL (e.g. Eppendorf BioPure®)

5.1.2 Pyrogen-free pipettes and barrier tips covering the range from 0.01 to 1 mL (e.g. Sarstedt Biosphere®)

5.1.3 Pyrogen-free dispenser tips, 100 µl increment (e.g. Eppendorf BioPure®)

5.1.4 Repeat pipettor or eight-chanel pipettor

5.1.5 96 well plate, pyrogen-free (e.g. Costar 3596, ACC PYROPLATE® or equivalent)

5.1.6 Disposable endotoxin-free glass dilution tubes 13 × 100 mm (Lonza N207) or 12 x 75 mm (ACC TB240) or equivalent

5.1.7 Gel-clot test tubes (ACC, TS050)

5.1.8 Microcentrifuge

5.1.9 Refrigerator, 2-8°C

5.1.10 Freezer, -20°C

5.1.11 Vortex mixer

5.1.12 Parafilm ® "M" Laboratory film (Pechiney Plastic Packaging)

5.1.13 Pipettes covering the range from 0.05 to 10 mL

5.2 Reagents

5.2.1 Test nanomaterial

5.2.2 Sodium hydroxide, 0,1 N, endotoxin free (e.g. Acila 1712200)

5.2.3 Hydrochloric acid 0,1 N, endotoxin free (e.g. Acila 1712300)

5.2.4 Control Endotoxin Standard, (Associates of Cape Cod (ACC), E0005)

5.2.5 LAL Reagent PYROTELL® For The Detection And Quantification Of Gram Negative Bacterial Endotoxins (PYROQUANT DIAGNOSTIK, Associates of Cape Cod, Inc. (ACC))

5.3.6 Glucashield® (1→3)-ß-D-Glucan Inhibiting Buffer (PYROQUANT DIAGNOSTIK GmbH, ACC)

5.2.7 LAL Reagent Water (PYROQUANT DIAGNOSTIK GmbH, ACC)

5.2.8 Endotoxin free water (e.g. ACC W0051 ; Acila 1715050; or equivalent)

5.3 Reagent Preparation

Store all provided reagents of the kit at 2 – 8°C. Prior to use allow reagents to equilibrate to room temperature[3].

5.3.1 Preparation of Pyrotell LAL Reagent

The assay reagent is provided as lyophilized mixture of LAL lysate It is reconstituted according to manufacturer's recommendations, and can be performed in LAL grade water (LAL Reagent Water) or Glucashield® Buffer available from the supplier as separate components. EUNCL preferred method is the application of Glucashield® buffer which allows to exclude interference from β-1,3-Glucans which is very common in nanomaterials produced using filtration steps. Substances containing β-1,3-Glucans are important sources of false-positives and a synergistic response (i.e. enhancement) is frequently seen with β-Glucan containing samples spiked with endotoxin. The usage of a Glucashield® buffer is therefore indicated whenever β-1,3-Glucan contamination is expected [3].

Reconstitute Pyrotell- LAL Reagent only before use. Add the volume as indicated on the vial label, which usually is 5 mL. In case for a larger number of samples more than one vial is required, pool reconstituted reagent of two or several vials before use. Exercise caution to avoid formation of air bubbles. Do not vortex the reconstituted lysate. Cover the vial with Parafilm M® when not in use.

Store the lyophilized Pyrotell- LAL Reagent at -20 to 8°C until expiration date on the label. Reconstituted LAL Reagent should be used promptly and is stable for up to 24 hours at 2 to 8°C., or can be stored at or below -20°C or colder for up to three months if frozen immediately after reconstitution. Freeze and thaw the reconstituted LAL Reagent only once (ACC) [3].

5.3.2 Endotoxin Control Standard Endotoxin stock solution

E.coli lipopolysaccharide (LPS) supplied by ACC is a USP certified control standard endotoxin (CSE) provided as a lyophilized powder. Prepare the stock solution of 1000 EU/mL by reconstitution of the Control-Standard Endotoxin (CSE, *E. coli* O55:B5 Endotoxin [3]).

Remove the metal seal from the vial, break the vacuum by lifting the stopper just enough to allow air to enter, and aseptically remove the stopper. Add LAL Reagent water directly and with caution to the CSE vial. The final volume needed for reconstitution of the CSE vial should be calculated for each lot and depends on product potency determined with a specific lot of LAL reagent relative to the current FDA or USP lot of reference. The specific volume needed to reach a potency of 1000 EU/mL can be calculated from the Custom Certificate of Analysis (provided by the supplier Associates of Cape Cod, Inc. [3]).

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. Vortex the CSE for at least 30 seconds each time immediately before taking an aliquot for usage to make appropriate dilutions.

The reconstituted stock CSE solution is stable for 4 weeks stored at 2-8°C, do not freeze CSE (Product Insert CSE Endotoxin *E. coli* 0113:H10, ACC) [3]. Before usage of the stored stock bring to room temperature and mix vigorously for 15 minutes in order to release endotoxin that tends to attach to the glass surface.

5.4 Assay Control Reactions

5.4.1 Preparation of Inhibition/Enhancement test using a Positive Product Control (PPC)

For the verification of results it is necessary to prepare samples with a defined amount of endotoxin standard to determine inhibition processes or interferences with the product. The nominal endotoxin concentration spiked in IEC are described in Measurement Procedure, "Bench Sheet" and Data Analysis.

5.4.2 Quality Controls

As a quality control a defined amount of endotoxin standard is spiked into endotoxin free water or respective diluent for samples. Details are described in Measurement Procedure, "Bench Sheet" and Data Analysis.

5.4.3 Negative Control

Use endotoxin free LAL reagent water or respective diluent buffer for the samples as a negative control reference.

Transfer 100 µL of each prepared Assay Control Reaction into the 96-well plate as directed by the assay template.

6 Procedure

6.1 General remarks

Most importantly microbial or endotoxin contamination of all samples and materials having contact with the sample and all used test reagents must be avoided by careful handling and technique.

6.2 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. It may be necessary to adjust the pH of the sample to within the range 6.0–8.0 using either sterile endotoxin-free sodium hydroxide or hydrochloric acid. 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 or other diluent, the diluent alone must be tested for endotoxin contamination in the assay. The concentration of nanomaterial is unique to each formulation. The goal of this test 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 from the stock using several dilutions not exceeding so called Maximum Valid Dilution (MVD).

To determine the MVD one needs to know three parameters: endotoxin limit (EL), sample concentration and assay sensitivity (λ). The EL is calculated according to the following formula: EL=K/M, where K is maximum endotoxin level allowed per dose (5EU/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 [5]. Note, estimation of EL for nanomaterials used as radiopharmaceutical or as medical device will be different[5]. 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 (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/m2 to mg/kg the dose in mg/kg is divided by the conversion factor of 37, indicated as km (for mass constant). The km factor has units of kg/m2; it is equal to the body weight in kg divided by the surface area in m2. Example 74 mg/m2 /37 = 2mg/kg [6].

The MVD is determined according to the following formula: MVD= (EL x sample concentration)/ λ). For example, when nanoparticle sample concentration is 10 mg/mL and it's maximum dose in mouse is 123 mg/kg, the HED is 123/12.3=10mg/kg; EL for all routes except intrathecal is 0.5 EU/mg (5EU/kg/10mg/kg) and MVD is 166.7 ((0.5 EU/mg x 10 mg/mL)/0.03 EU/mL). In this case, the nanomaterial will be tested directly from stock and at several dilutions not exceeding 166.7, e.g. 5, 75 and 150 (or 166). When the information about the dose is unknown, the highest final concentration of the 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.

6.3 Measurement Overview

The gel clot LAL procedure described here follows the USP BET 85 protocol. For complete details on this procedure, please consult the USP reference 5. Outlined below is a "Bench Sheet" that can be printed and used alongside the USP protocol.

Briefly, the test includes three steps: Step 1, Confirmation of Labeled Lysate Sensitivity; Step 2, Test for Interfering Factors; and Step 3, Endotoxin assessment in the test sample by either limit test or quantitative test. Step 1 can be done once and need not to be repeated until bacterial endotoxin standard and lysate lots have changed. Step 2 analysis 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 the Step 3 is done only after absence of interference has been confirmed in the Step 2.

The qualitative (limit) test results are negative when both replicates did not clot. If clotting was observed in one replicate the test has to be repeated. If in the repeated test one or both replicates clotted, the sample contain endotoxin contamination at the level equal to or more than the assay sensitivity. If the diluted sample was tested, the assay sensitivity has to 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 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.

6.4 Measurement Procedure

6.4.1 Label as many reaction tubes as needed to accommodate the number of analyzed test samples. Refer to bench sheet for details about number of replicates used in step 1, step 2 and step 3 of the assay

6.4.2 Aliquot 100 µL of water, controls or test sample per tube

6.4.3 Prepare CSE such as 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

6.4.4 Add 100 µL of lysate per test tube, vortex briefly, then place entire rack into a water bath set to 37 °C for 1 h

6.4.5 Remove samples from water bath and dry using paper towel

6.4.6 Invert the tube using smooth motion and record results using "+" (firm clot) or "-" (no clot or loose clot) on the bench sheet

6.4.7 Proceed with analysis according to the USP BET 85, use bench sheet as supporting material

Note: During the measurement time, do not disturb the reaction tubes. The laboratory bench supporting the incubator water bath should be free from excessive vibration.

6.5 Measurement Procedure: "Bench Sheet" and Data Analysis

6.5.1 Step 1 - Qualification of Reagent Sensitivity (Do 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 into 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 solutions 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 (∑e/f), where ∑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**

6.5.2 Step 2 – Inhibition Enhancement Control

Important Note: This step should be repeated for each nanoparticle concentration. Ideally undiluted sample is tested first. If interference is found for undiluted sample, repeat step 2 with as many dilutions as necessary to overcome the interference, but make sure the dilution does 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 data_____or NCL PCC_____

To prepare samples B1 and C1 spike CSE at a final concentration 2λ into nanoparticles and LAL water, respectively; then perform 3 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. The 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**	**-**

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 formula described in Section 3 of Step 1. Record the data below:

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

Using data from the above table (see section 3 of step 2) and calculation of geometric mean sensitivity to 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 interfering 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 Step 3A or 3B. Choice between 3A and 3B depends on the project need.

6.5.3 Step 3A – Limit Test

Important Note: This test is done at highest nanoparticle concentration (lowest dilution of the stock nanoparticle sample) not interfering with gel-clot LAL. Refer to Step 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 dilution_____or at MVD_____

Nanoparticle stock concentration_____mg/mL by API_____total_____other_____

Concentration is based on client's data_____or NCL PCC_____

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 (see section 2 of step 3A) 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**

6.5.4 Step 3B – Quantitative Test

Important Note: This test is done at highest nanoparticle concentration (lowest dilution of the stock nanoparticle sample) not interfering with gel-clot LAL. This dilution is called "initial dilution". Refer to Step 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 part 2, for purposes of this test it is called "initial dilution"; subsequent dilutions of the initial dilution should be done in a way such as the final dilution not exceeding 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 is within the MVD. Likewise, if the initial dilution is 40, then subsequent dilution 8 will be above the MVD.

3. Result Evaluation

3.1 Calculate geometric mean end point concentration for sample C according to formula described in Section 3 of Step 1. Use the table in section 3.2 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	

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

3.3.1 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 = _____**

3.3.2. 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 intermediate dilution analyzed in the assay.

- Record endpoint concentration here_____ x dilution factor = _____EU/mg

- If none of the dilutions of the test sample is positive in a valid assay, report the endotoxin concentration as < λ_____

- If diluted sample was analyzed report concentration as < λ x lowest dilution factor_____

- If all dilutions are positive the endotoxin concentration is ≥ λ x initial dilution factor x 8_____

7 Acceptance Criteria

The preparation under test meets the requirements of the test if the concentration of endotoxin in both replicates is less than that specified in the individual monograph [3].

8 Health and Safety Warnings, Cautions and Waste Treatment

Use Pyrotell-T for in vitro diagnostic purposes only. Do not use it for the detection of endotoxemia. The toxicity of the reagent has not been determined; thus, caution should be exercised when handling Pyrotell-T.

Inform yourself about the content and sample material and all relevant safety issues concerning the samples before unpacking and handling of any received sample.

Always wear adequate personal protective equipment, in particular protective laboratory coat, gloves and safety glasses and take all necessary precautions to protect yourself and others. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with the products. Use respiratory protection whenever advisable. Open the sample vials only under sterile conditions of the sterile work bench in order to avoid sample spilling and contamination. Take all necessary precautions to avoid any further sample spilling in case of damaged sample container. Waste disposal has to be proceeded in a proper form

using means adequate for the material specifications, in compliance with the laboratory regulations and general regulatory conditions according to applicable legal regulations.

9 Abbreviations

API active pharmaceutical ingredient

BW blank water

CSE control standard endotoxin

CV coefficient of variation

EL Endotoxin Limit

EU endotoxin unit

FDA Food and Drug Administration

HCl hydrochloric acid

IEC inhibition/enhancement control

LAL Limulus Amebocyte Lysate

MVD Maximum Valid Dilution

NaOH sodium hydroxide

PBS phosphate buffered saline

PES polyethersulfone

PPC positive product control

RT room temperature

10 References

1: Bang, F.B. A bacterila disease of *Limulus polyphemus*. Bull. Johns Hopkins Hosp. 98:325 (1956)

2: Levin, J., Bang. F.B. Clottable protein in *Limulus*: its localisation and kinetics of its coagulation by endotoxin. Thromb. Diath. Haemorrh. 19:186 (1968)

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

4: FDA Guidance for Industry and Reviewers Estimating the Safe Starting Dose in Clinical Trials for Therapeutics in Adult Healthy Volunteers. December 2002

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