Detection and Quantification of Gram Negative Bacterial Endotoxin Contamination in Nanoparticle Formulations by Kinetic Chromogenic LAL Assay

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1.0	13.07.2016	All	Initial Document	Rainer Ossig
1.1	01.10.2016	Chapter 6	Acceptance criteria are now in line with	Matthias Rösslein

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Table of Content

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. Principle of the Method

This method relies on an *in vitro* end-product endotoxin test which utilizes a preparation of *Limulus Amebocyte* Lysate (LAL), to detect endotoxin photometrically with an incubating temperature controlled microplate reader.

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]. The method is based the initial reaction of the LAL with endotoxin. A LAL proenzyme is activated which is further used in this assay to cleave a synthetic chromogenic substrate that releases the yellow coloured p-nitroaniline. The occurrence of the color is measured photometrically at 405 nm throughout the incubation period. The amount of endotoxin in the sample is determined by the time required for the appearance of the yellow color. The amount of endotoxin present which is calculated from a standard curve prepared by dilution of an endotoxin standard of known concentrations of into LAL grade water.

2 Applicability and Limitations (Scope)

This SOP was developed to determine and quantify endotoxin contamination of different nanomaterials. This SOP was created according to ISO 297014 adapted for the analysis nanomaterials[4].

3 Related Documents

Table 1:

Document ID	Document Title
NCL Method STE-1.4	**Detection and Quantification of Gram Negative Bacterial Endotoxin Contamination in Nanoparticle Formulations by Kinetic Chromogenic LAL Assay**

4 Equipment and Reagents

4.1 Equipment

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

4.1.2 Micropipettes covering the range from 0.01 to 1 mL (e.g. Eppendorf)

4.1.3 Pyrogen-free filter tips for Micro-Pipettes covering the range from 0.01 to 1 mL (e.g. Sarstedt Biosphere®)

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

4.1.5 Dispenser (Repeat pipettor) or eight-chanel pipettor (e.g. Eppendorf)

4.1.6 96 well plate, pyrogen-free (e.g. Costar 3596)

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

4.1.8 Reagent reservoirs (Lonza 00190035 or equivalent)

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

4.1.10 Microcentrifuge

4.1.11 Refrigerator, 2-8°C

4.1.12 Freezer, -20°C

4.1.13- Vortex mixer

4.1.14 automated Microplate reader, temperature controlled 37°C, 405 nm absorption
(e. g. BMG Novostar, ClarioStar)

4.2 Reagents

4.2.1 Test nanomaterial

4.2.2 Limulus Amebocyte Lysate (LAL) kinetic chromogenic Kit (e.g. Kinetic-QCL™ Lonza 50-650U), including LAL Reagent, Control Standard Endotoxin and LAL Reagent Water

4.2.3 Beta-Glucan-Blocker Kit (e.g. Lonza N190)

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

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

4.2.6 Endotoxin free water (either part of the kit or separately ordered, e.g. Acila 1715050)

4.3 Reagent Preparation

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

4.3.1 Preparation of LAL Reagent

The assay reagent is provided as lyophilized mixture of the Limulus Amebocyte Lysate (Kinetic-QCL™ Reagent, Yellow-Labeled Vial). It is reconstituted according to manufacturer's recommendations, performed in LAL grade water (LAL Reagent Water) available from the reagent kit or separately purchased.

Reconstitute only immediately before use. Most reagent vials will require reconstitution to a final volume of 2.6 mL which is good for 24 single reactions. In case for a larger number of samples more than one vial is required, pool the reconstituted Lysate of two or several vials before use. Do not vortex the reconstituted lysate. Mix by gently swivel to avoid foaming. In case of bubbles allow to clear before use.

Store the lyophilized LAL Reagent at 2–8°C. After reconstitution the LAL Reagent should be used promptly and is stable for 8 hours at 2–8°C. Reconstituted LAL reagent can be stored at -20°C or colder for up to two weeks. Freeze and thaw the reconstituted LAL Reagent only once[3] .

4.3.2 Endotoxin Control Standard Endotoxin stock solution

Prepare a 50 EU/mL Endotoxin stock solution by reconstitution of the Control Standard Endotoxin supplied in the kit (*E. coli* O55:B5 Endotoxin, Red-Labeled Vial). Add LAL Reagent water directly to the vial. The specific volume needed to reach a solution containing 50 EU/mL is given by the Certificate of Analysis of the supplier. Vigorously mix the vial for at least 15 minutes on a vortex mixer. The stock solution is stable for 4 weeks at 2-8°C [3] and should be stored in the original vial. Before usage always bring the stock to room temperature and mix vigorously for 15 minutes in order to release endotoxin that tends to attach to the glass surface of the vial.

4.3.3 Preparation of Endotoxin calibration standards

The assay has been optimized to provide linear response from 0.005 EU/ml to 50.0 EU/mL. However, in order to adjust the quantitative range of an individual test the user may choose to truncate the standard curve depending on specific product. A minimum of three standards is required[3].

Label disposable pyrogen-free glass dilution tubes for the endotoxin dilutions. Prepare a serious of dilutions by adding 0.1 mL of the prior endotoxin solution into 0.9 mL of LAL Reagent Water. Each dilution should be vigorously vortexed for at least 1 minute before proceeding with the next step of the dilution series.

The endotoxin calibration standards are prepared as described in the following table (alternative dilution schemes may be used):

Dilution scheme: Preparation of a series of endotoxin dilutions for calibration standards

Sample	Nominal Concentration [EU/mL]	Preparation Procedure
Cal. 1	5.0	100 µL of 50 EU/mL stock solution + 900 µL LAL reagent water
Cal. 2	0.5	100 µL Cal. 1 + 900 µL LAL reagent water
Cal. 3	0.05	100 µL Cal. 2 + 900 µL LAL reagent water

Cal. 4	0.005	100 µL Cal. 3 + 900 µL LAL reagent water

Each sample should be vigorously vortexed for at least one minute prior to use.

4.4 Assay Control Reaction

4.4.1 Preparation of Inhibition/Enhancement Control test using a positive product control (PPC)

For the verification of assay results it is necessary to prepare samples containing a defined amount of endotoxin standard to determine potential inhibition processes or interferences with the assay procedure. Therefore 50 µL of a 5 EU/mL standard solution are added to 450 µL nanoparticle suspension of the sample, resulting in a spiked endotoxin concentration of 0.5 EU/mL. The concentration of nanoparticles should be equal to one assayed in test-sample. You will need to prepare IEC for each dilution of the nanomaterial assayed in this test. A positive control using the same endotoxin standard concentration in LAL Reagent Water will be used as Quality Control for the assay reaction. Endotoxin free LAL reagent water or the respective diluent buffer is used as a negative control reference. Transfer 100 µl of each IEC solution, Negative Controls and Quality Control into the 96-well plate as directed by the assay template.

The endotoxin Control Reactions are prepared as described in the following tables (alternative dilution schemes may be used) :

Dilution scheme: Preparation of the PPCs with defined amount of endotoxin standard

Sample	Nominal Concentration [EU/mL]	Preparation Procedure
Int. A	5.0	50 µL of 50 EU/mL Stock + 450 µL LAL reagent water
IEC	0.5	50 µL Int. A + 450 µL nanoparticle suspension

Each sample should be vigorously vortexed for at least one minute prior to use. Intermediate solution A is prepared only to make controls and is not used in assay.

Dilution scheme: Preparation of Quality Control with defined amount of endotoxin standard

Sample	Nominal Concentration (EU/mL)	Preparation Procedure
Int. A	5.0	50 µL of 50 EU/mL Stock + 450 µL LAL reagent water
QC	0.5	50 µL Int. A + 450 µL LAL reagent water

Each sample should be vigorously vortexed for at least one minute prior to use. Intermediate solution A is are prepared only to make controls and is not used in assay.

4.4.2 Alternative On Plate Method for of Inhibition/Enhancement Controll

Pipette 90 µL of test sample (or dilution) into the intended wells for PPC of the 96-well plate, as directed by the assay template. Subsequently add 10 µL of the 5.0 EU/mL endotoxin standard solution to these wells to reach a 0.5 EU/mL concentration of Endotoxin. Mix gently by tapping the side of the plate.

4.4.3 Negative Control

Endotoxin free LAL Reagent Water or diluent buffer is used 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.

5 Procedure

5.1 General remarks

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

5.2 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. Pyrogen-free Tris buffer may also be used to prepare samples for endotoxin detection in place of water as a sample diluent to adjust pH of highly acidic or basic samples. To avoid sample contamination always measure the pH of an aliquot of the prepared sample. 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 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). 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 (1). 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 guidelines for other conversion ratios [6]. 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 km (for mass constant). The km factor has units of kg/m^2; it is equal to the body weight in kg divided by the surface area in m2. Example 74 mg/m^2 /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 1000 ((0.5 EU/mg x 10 mg/mL)/0.005EU/mL). In this case, the nanomaterial will be tested directly from stock or at several dilutions not exceeding the MVD of 1000, e.g. 10, 100 and 1000 times dilution. When the information about the dose is unknown, the highest final concentration of the test nanomaterial is 1 mg/mL, which is used to calculate the MVD. 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.

Substances containing known as β-1,3-Glucans are important sources of false-positives and a synergistic response (i.e. enhancement) is frequently seen with β-Glucan samples spiked with endotoxin. The usage of a β-G-Blocker is indicated in cases where β-1,3-Glucan contamination is suspected. The β-G-Blocker can be applied to the sample dilution according to the manufacturers instructions. Add one volume part of β-G-Blocker to each sample volume. To avoid excessive dilution rates perform the preceding dilution to only twice the test concentration that is required finallyand mix. Afterwards add one volume of β-G-Blocker to reach final sample dilution. Vortex this sample solution and use for preparation of the PPCs (Inhibition/Enhancement controls) or dispense directly to the wells of a 96-well plate as test samples as described before.

5.3 Flow chart

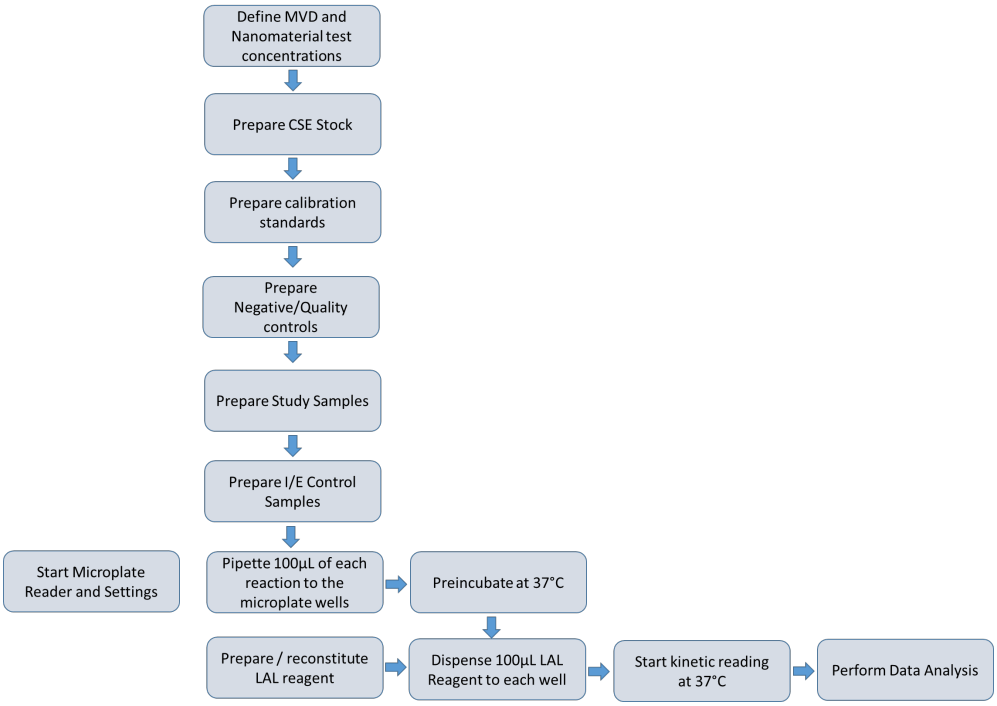

Figure 1: Brief outline of the workflow

5.4 Measurement Procedure

5.4.1 Test procedure

Turn on the reader instrument approximately 20-30 minutes before starting the assay to allow the instrument to warm up. Set reader temperature to 37 °C. Select detection wavelength to 405nm, and adjust all settings as listed below, or use a preinstalled program according to these settings.

Setting for automated temperature controlled Microplate reader (e.g. BMG Clariostar, Novostar):

405 nm

37 °C

40 cycles, 150 s cycle time (6000 s total reading time)

(Alternatively, shorter cycle times with higher number of performed cycles may be used with the total reading time of at least 6000 s)

Dispense 100 µL of prepared endotoxin standards, different product sample dilutions (S), product inhibition samples (PPC), Quality control (Qc), and blank Endotoxin free LAL reagent water or diluent buffer (BW) in the wells of the 96 well plate. Prepare each sample at least in duplicates.

The following matrix can be used as an example template for pipetting.

Other plate designs may be advantageous to apply for different sample and control reaction numbers.

1	2	3	4	5	6	7	8	9	10	11	12
	5	5	0,5	0,5	0,05	0,05	0,005	0,005	BW	BW	
	S1.1	S1.1	S1.2	S1.2	S1.1 PPC	S1.1 PPC	S1.2 PPC	S1.2 PPC	Qc	Qc	
	S2.1	S2.1	S2.2	S2.2	S2.1 PPC	S2.1 PPC	S2.2 PPC	S2.2 PPC	Qc	Qc	
	S3.1	S3.1	S3.2	S3.2	S3.1 PPC	S3.1 PPC	S3.2 PPC	S3.2 PPC	Qc	Qc	
	S4.1	S4.1	S4.2	S4.2	S4.1 PPC	S4.1 PPC	S4.2 PPC	S4.2 PPC	Qc	Qc	
	S.5.1	S5.1	S5.2	S5.2	S5.1 PPC	S5.1 PPC	S5.2 PPC	S5.2 PPC	Qc	Qc	

After pipetting of all samples and controls is performed, preincubate the microplate with its cover on for at least 10 minutes at 37 ± 1°C in the spectrophotometer to adjust temperature of all reaction samples.

During this time reconstitute the necessary LAL Reagent vials with 2.6 ml LAL Reagent Water per vial (according to the manufacturers instruction), mix gently (do not vortex) but thoroughly as described above (for details read under -Reagent Preparation-).

After 10 minutes of incubation remove the microtiter plate from the incubation chamber. Use a dispenser to immediately add 100 µL reconstituted LAL- Reagent into each of the reaction wells. Work quickly but careful to avoid causing bubbles in the well. Control all wells for absents of bubbles and start automated measuring procedure immediately (reading is performed with the microplate cover removed).

5.5 Data Analysis

The reaction time needed for the appearance of yellow colour is inversely proportional to the amount of present endotoxin. For the determination of the exact endotoxin amount it is necessary to create a standard curve of at least 3 to 4 different concentrations (e. g. 5 EU/mL, 0.5 EU/mL, 0.05 EU/mL, 0.005 EU/mL). Based on the values for the endotoxin standard curve a log/log linear correlation is used to calculate values of the corresponding Endotoxin concentration from the reaction time in EU/mL. The initial absorbance of each well is used as blank for its subsequent readings to perform a baseline correction and to determine the time to reach an increase of 0.200 absorbance units. The correlation coefficient for the standard curve should be ≥0.980 to enable a reliable interpolation of the unknown samples. The different parameters are the absorption values for the x-range, mean reaction time for the y-range, and 0.200 as threshold value, in order to determine the reaction time for the increase of 0.2 absorbance units. The Excel Add-in xlAutomation.Trend can be used, or a respective template for the MARS Data Analysis Software (BMG Labtech) is used to directly calculate the results from the microplate reader.

The recovery rate of positive product control (PPC) and Quality controls (QC) is calculated by dividing the measured spiked endotoxin concentration by the nominated one to determine potential inhibition or enhancement reactions of the sample ingredients at the respective concentration of the tested sample.

6 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 6.1, run should berepeated.

5. If standard curve fails to meet acceptance criterion described in 6.1 – 6.3, 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% [4]. 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 5.

7 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 5 for details).

8 Health and Safety Warnings, Cautions and Waste Treatment

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 that further sample spilling is avoided 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

CV coefficient of variation

EU endotoxin unit

HCl hydrochloric acid

LAL Limulus Amebocyte Lysate

MVD Maximum Valid Dilution

NaOH sodium hydroxide

PBS phosphate buffered saline

PES polyethersulfone

PPC positive product control

RT room temperature

S sample

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.