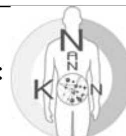
Responsible partner for this SOP:	NanoKon- INM	Project Logo: 

Title: **Detection and semi-Quantification of Endotoxin Contaminations in Nanoparticle Suspensions**

Subtitle: ***Limulus* amebocyte lysate (LAL) Gel Clot Assay**

AUTHORED BY:	DATE:
Kucki, Melanie	25.06.2012

REVIEWED BY:	DATE:

APPROVED BY:	DATE:

DOCUMENT HISTORY

Effective Date	Date Revision Required	Supersedes

Version	Approval Date	Description of the Change		Author / Changed by
001				

TABLE OF CONTENT

1 Purpose

2 Objective

3 Regulatory basis, reference documents

4 Related documents

5 Definitions

6 Procedure

7 Scope / area of application

8 Attachments

9 Health, safety and environmental considerations

1 PURPOSE

In vitro endotoxin test for the detection and semi-quantification of endotoxin contaminations in aqueous nanoparticle suspensions.

2 OBJECTIVE

Endotoxin contamination of engineered nanoparticles is a critical factor. Even low endotoxin concentration can induce a response of the innate immune system. If undetected, endotoxin contamination in nanoparticle formulations can cause misleading results in *in vitro* tests and influence the general assessment of nanoparticle safety.

The LAL Gel Clot Assay is a semi-quantitative test for the detection of biological active lipopolysaccharides (LPS, Endotoxins). The *Limulus* amebocyte lysate (LAL) is prepared of the hemolymphe of the horseshoe crab *Limulus polyphemus*. It contains all components of a coagulation cascade, which can be triggered by activation of the LPS-sensitive serine protease Factor C. The presence of endotoxins induces a series of enzymatic reactions resulting in the hydrolyzation of the clotting protein coagulogen to coagulin, which self-associates and forms a gel clot.

3 REGULATORY BASIS, REFERENCE DOCUMENTS

This assay protocol follows and is based on the information given in the following documents, but it is not strictly in accordance with each document in every point:

[1] European Pharmacopoeia Monograph 2.6.14 Bacterial Endotoxins, BET

[2] USP 34-NF29 <85> Bacterial Endotoxins. Rockville, MD, United States

[3] ISO 29701 Nanotechnologies - Endotoxin test on nanomaterial samples for *in vitro* systems - *Limulus* amebocyte lysate (LAL) test, 2010

[4] Manufacturer's Instructions LAL Gel Clot Assay (Lonza, Limulus Amebocyte Lysate (LAL) Pyrogen Plus)

[5] Manufacturer's Instructions ß-Glucan Blocker (Lonza)

[6] Dobrovolskaia M., Neun B., 2011; Protocol 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, MD, USA

[7] Dobrovolskaia M., Neun B., 2011; Protocol NCL Method STE-1.1 Detection and Quantification of Gram Negative Bacterial Endotoxin Contamination in Nanoparticle Formulations by End Point Chromogenic LAL Assay, Nanotechnology Characterization Laboratory, National Cancer Institute-Frederick, MD, USA

[8] Dawson M.E., 2005: Interference with the LAL Test and How to Address it, LAL Update, Associates of Cape Cod, Incorporated, Vol. 22 (3): 1-6

[9] Vallhov H., Qin J., Johansson S.M., Ahlborg N., Muhammed M.A., Scheynius A., Gabrielsson S., 2006: The Importance of an Endotoxin-Free Environment during the Production of Nanoparticles Used in Medical Applications, Nano Letters, Vol. 6 (8):1682-1686

[10] Dobrovolskaia M.A., Germolec D.R., Weaver J.L., 2009: Evaluation of nanoparticle immunotoxicity, Nature Nanotechnology 4: 411-414

[11] Neun B.W., Dobrovolskaia M.A., 2011: Detection and Quantitative Evaluation of Endotoxin Contamination in Nanoparticle Formulations by LAL-based Assays, in Scott E. McNeil (ed.), Characterization of Nanoparticles Intended for Drug Delivery, Methods in Molecular Biology, Vol. 697, 121-130

4 RELATED DOCUMENTS

Table 1: *References to documents needed to proceed according to this procedure*

Document ID	Document Title

5 DEFINITIONS

Table 2: *Glossary of Terminology used in the SOP*

Term	Description
CSE	Control standard endotoxin
EU	Endotoxin unit, 1 EU = 1 IU (International Unit) [1]
Factor C	LPS-sensitive serine protease, triggers coagulation cascade upon activation by LPS
Factor G	Glucan-sensitive protein which triggers coagulation cascade
IEC	inhibition/enhancement control
LAL	Limulus amebocyte lysate
Lambda (λ)	sensitivity of the assay in EU/ml
LPS	Lipopolysaccharide, Endotoxin
MVD	maximum valid dilution, maximum allowable dilution of a sample at which the endotoxin limit can be determined [1]
USP	United States Pharmacopoeia

6 PROCEDURE

a) Short Description

Test samples, standards, and controls are mixed 1:1 with reconstituted LAL and incubated for 1 hour at 37°C. After incubation test vessels are carefully inverted 180°. A positive reaction is characterized by the presence of a firm gel clot after inversion. A negative reaction is characterized by the absence of a solid clot and the presence of a liquid.

b) Materials and devices

- Sterile nanoparticle suspension with a particle concentration > 1 mg/ml
- Endotoxin-free Sodium hydroxide (NaOH) / Hydrochloric acid (HCl) for pH adjustment if necessary
- CSE - Control Standard Endotoxin (e.g. *Escherichia coli* Serotype 055:B5)
- Endotoxin-free LAL reagent water or endotoxin-free Millipore Q
- Lyophilized LAL
- ß-Glucan Blocker (Lonza) or related product
- Endotoxin-free dilution and reaction vessels
- Pipettes and endotoxin-free pipette tips
- Sterile work bench (SWB)
- Tube racks
- Vortex mixer
- Non-circulating hot water bath (37°)
- Timer
- Refrigerator (2-8 °C) and Freezer (-20°C) for reagent storage

All glass or other lab ware that come in contact with the test samples, controls and LAL reagent have to be sterile and endotoxin-free.

c) Sample preparation

Sample preparation and all other actions prior to transfer of the samples to the water bath are performed in a sterile work bench (SWB) to avoid microbiological contamination.

- Control pH of a sample aliquot; valid pH range: 6.0-8.0 (Do not use this aliquot for further endotoxin testing!)
- Adjust pH of the nanoparticle suspension by addition of endotoxin-free NaOH or HCl if necessary

- Dilute nanoparticle suspension to a standard particle concentration of 1 mg/ml [6, 7]
- Store sterile nanoparticle samples at 4-8°C until use.

d) Detailed description of the Project

Preparation

a) Reconstitution of the Control Standard Endotoxin (CSE)

- Reconstitute the Control Standard Endotoxin (CSE) with LAL reagent water (reconstitution volume and storage conditions are given by the manufacturer)
- Vortex for at least 15 minutes
- Dilute an aliquot of the endotoxin to a concentration of 1 EU/ml in LAL reagent water

b) Reconstitution of the LAL

- Reconstitute the LAL with LAL reagent water (reconstitution volume is given by the manufacturer)
- Swirl for at least 30 seconds. Do not shake!
- Use reconstituted LAL as soon as possible or store under appropriate conditions (up to 24 hours at 4-8°C, up to four weeks at -10 to -20 °C)
- Do not use the LAL if the colour has turned yellow or in case of precipitation and increase of turbidity.

General test procedure

- Allow all samples and reagents used to equilibrate to room temperature
- Prepare 2 replicates of each sample/control with 100 µl volume
- Add 100 µl reconstituted LAL to 100 µl sample/control
- close or cap the reaction vessel
- Vortex for 1 second
- Place tubes in a 37°C non-circulating water bath

- Incubate for 1 hour (Do not remove or disturb tubes during incubation!)
- Take tubes carefully out of the water bath
  (Note: avoid vibrations and shocks! Tubes have to be handled with care due to mechanical instability of the forming gel clots!)
- Read out the assay by inverting tubes 180°:
  - Positive reaction: firm gel clot after inversion
  - Negative reaction: absence of a solid clot after inversion
- The end-point is the last positive results in the dilution series

- The assay is performed in duplicate with 2 replicates each (in contrast to [1]. Each run is made with a new dilution series of the sample.

STEP 1: Perform **Control of the labelled assay sensitivity:**

Prior to endotoxin detection the labelled sensitivity of the assay (given by the manufacturer) has to be controlled. This check has to be performed once for each LAL lot applied or if the lot of the CSE has changed.

- Use the 1 EU/ml endotoxin solution to prepare a serial two-fold dilution series that brackets the labelled LAL sensitivity: 2λ, 1λ, 0.5λ, and 0.25λ.
- Negative control: endotoxin-free reagent water
- Perform the general test procedure (see above)
- Calculate the geometric mean sensitivity of the assay is by:

$$\text{Antilog } \Sigma_{\text{log sensitivity}} / \text{number of replicates} = x \text{ EU/ml}$$

- the calculated geometric mean should be in accordance to the labelled sensitivity

STEP 2: Perform **Quantification of Endotoxin:**

- Calculate the MVD for the Assay:

$$\text{MVD} = \text{sample concentration} * \text{Endotoxin limit} / \text{assay sensitivity } (\lambda)$$

- In this protocol nanoparticle suspensions are treated as medical devices (Endotoxin limit 0.5 EU/ml), due to the fact that for most nanoparticle suspensions no data for human or animal doses is available [7].
- For an assay sensitivity of 0.03 EU/ml and a sample concentration of 1 mg/ml the MVD is 16.
- Use the nanoparticle sample (1 mg/ml particles) to prepare a serial two-fold dilution series (1/2, 1/4, 1/8, 1/16) in LAL reagent water (not further than the MVD!)
- Use the 1 EU/ml endotoxin solution to prepare a positive control sample of 2 λ, here 0.06 EU/ml
- Use endotoxin-free LAL reagent water as negative control sample
- Perform the general test procedure (see above)
- Calculate the endpoint concentration by

Endpoint concentration = Endpoint * assay sensitivity (λ)
(The Endpoint is the highest dilution factor that yields a positive result.)

- If no dilution of the sample is positive, the endotoxin concentration in the test sample is reported to be below the assay sensitivity (λ)
- If all dilutions of the sample are positive, the endotoxin concentration in the test sample is reported to be equal or greater than the highest dilution factor multiplied by the assay sensitivity (λ)

STEP 3: Perform **IEC - Inhibition Enhancement Control**

To detect possible inhibition and enhancement, a two-fold dilution series of an endotoxin standard similar to STEP 1 is used, with the difference of using the nanoparticle suspension as diluent instead of reagent water.

- Use the 1 EU/ml endotoxin solution to prepare a serial two-fold dilution series that brackets the labelled LAL sensitivity: 2λ, 1λ, 0.5λ, and 0.25λ.
- Negative control: endotoxin-free reagent water
- Perform the general test procedure (see above)
- Calculate the geometric mean sensitivity of the assay is by:

Antilog $\Sigma_{\text{log sensitivity}}$ / number of replicates = x EU/ml

- the calculated geometric mean should be in accordance to the labelled sensitivity

Inhibition leads to under-estimation, enhancement to over-estimation of the endotoxin concentration. The test is valid if the detected sensitivity is in the range of 0.5λ and 2λ.
Note: Other protocols prefer to perform the IEC before the quantification test. But the IEC can only be performed if there is no detectable endotoxin within the non-spiked nanoparticle sample. So information about the presence of detectable endotoxin in the sample is necessary.
The results of the IEC are only valid for the particle concentration tested in the IEC! Other particle concentrations have to be tested separately.
The test is valid if the detected sensitivity (highest dilution factor with positive result) is in the range of 0.5λ and 2λ. It is not valid if it is below or above this region.

STEP 4: Perform **ß-1,3-Glucan Interference Test** (if Endotoxin specificity is needed!)

If endotoxin contamination is detected, the ß-1,3-Glucan interference test is performed to exclude interference with ß-1,3-Glucan. ß-1,3-Glucan can trigger the coagulation cascade by the activation of Glucan-sensitive Factor G. If undetected, the presence of ß-1,3-Glucan leads to an overestimation of the endotoxin content and false-positive results. Here ß-G-Blocker (Lonza) is used to block the reactivity of LAL to ß-1,3-Glucan:

- Dilute nanoparticle samples to twice the normal test concentration
- Mix ß-G-Blocker 1:1 to the nanoparticle sample (normal test concentration is reached)
- Perform assay as usual
- Controls: a) nanoparticle sample without ß-G-Blocker,
  b) ß-G-Blocker 1:1 in LAL reagent water,
  c) Positive control Endotoxin (2 λ) with ß-G-Blocker

e) Controls

f) Data analysis

g) Testing errors

Negative control is positive: endotoxin contamination of the a) reagent water or b) the reconstituted LA lysate or c) the reaction vessel

Positive control is negative: a) pH of the sample is out of optimal pH range of the assay. Check and adjust the pH if necessary.
b) LPS stock is degraded even if the specified storage duration is not expired. Repeat the experiments with fresh LPS stock.

LPS-spiked sample is negative: a) pH is out of range, b) LPS is degraded or c) assay inhibition by sample constituents

Interfering factors:

In general interference can result from factors that alter the LPS aggregate structure, bind to LPS or disturb the normal enzymatic activity of the LAL coagulation cascade. In several cases interference can be overcome by dilution of the sample.
Examples of interfering factors are citrate and EDTA, metall-chelators which are also used as stabilizing agents in nanoparticle suspensions. Chelation of Ca^{2+}-Ions can influence the LPS aggregation state or interferes with the Ca^{2+} dependent LAL coagulation cascade. Detergents like Triton-X and SDS (sodium dodecyl sulphate) can affect the LPS aggregation structure as well as the enzyme activity. Other interfering factors are certain blood constituents, e.g. albumin, LBP (LPS-binding protein), BPI (bactericidal/permeability-increasing protein), hemoglobin, synthetic and natural cationic amphiphilic peptides, lipoproteins, e.g. LDL, HDL, chylomicrons, as well as the serine protease trypsin and traces of organic solvents.

7 SCOPE / AREA OF APPLICATION

For the detection of endotoxins in aqueous nanoparticle suspensions, especially those that show optical interference with assays based on optical read-out (absorption, fluorescence). Examples are nanoparticles with high intrinsic absorption within the UV-vis region, e.g. iron-oxide, silver, gold, and barium sulphate or fluorescent labelled nano and- microparticles. Inhibition/enhancement controls have to be performed for every sample, for every nanoparticle type and surface modification. Up to date there is no LAL based assay format available that can be used for every nanoparticle formulation without showing interference events.

Endotoxin tests on nanoparticles suspended in buffers with high ionic strength or cell culture medium with and without FBS (fetal bovine serum) suffer from additional challenges and are not part of this SOP.

8 ATTACHMENTS

9 HEALTH, SAFETY AND ENVIRONMENTAL CONSIDERATIONS

Follow standard safety operating procedures and local lab rules. Wear appropriate protective equipment, gloves, protective clothing and eyewear. Endotoxins should be treated as potentially toxic. Sample preparation and all other actions prior to incubation are performed in a sterile work bench. Collect and dispose all waste in accordance with local laws.