PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Guidance Document for Endotoxin Testing

This is a SOP used by members of PATROLS only

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Authored by:
Dr. Roman Lehner, MSc. Hana Barosova, Prof. Barbara Rothen-Rutishauser
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Reviewed by:e
Enter here all reviewers and affiliations (e.g. J. Smith1) of the protocol.

Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	DD/MM/YYYY	Initial Document	

Table of Contents

1 Introduction:

Endotoxin (also known as lipopolysaccharide (LPS)) is a molecule found in the outer membrane of Gram-negative bacteria. It can initiate a strong immune response and serves as an early warning signal of bacterial infection [1]. The binding of LPS initiates the aggregation of different intracellular signaling proteins leading to cytokine production and the initiation of inflammatory signaling. Within this protein binding cascade, Toll-like-receptor 4 (TLR4) is the key receptor involved in LPS recognition and signal initiation in addition to the co-receptors CD14 and MD2 [2]. It has been shown that already very low levels of 0.1 EU/mL endotoxin can upregulate the expression of inflammatory genes (e.g. upregulation of the inflammatory IL1β gene) in primary human monocytes [3, 4]. The Food and Drug Administration (FDA) redommends a limit of 0.5 EU/mL for medical devices [5]. As endotoxin can easily bind to the surface of nanomaterials it is important to include endotoxin tests as described elsewhere [6, 7] since the presence of LPS in the nanomaterial suspensions can result in an induction of inflammation that is wrongly attributed to the nanomaterial [8, 9].

1.1 Scope and limits of the protocol

This guideline describes the endotoxin testing of nanomaterial suspensions intended for cell-based *in vitro* biological test systems by PATROLS. Depending on the physico-chemical characteristics such as the optical density, the materials could pose a significant problem of interference with both the components and the detection readouts which has to be considered for the planning of the experiments and the analysis.

2 Terms and Definitions:

Endotoxin

Part of the outer membrane of the cell envelope of Gram-negative bacteria

Note 1 to entry: The main active ingredient is lipopolysaccharides (LPS).

[SOURCE : ISO 29701:2010, definition 2.3]

Endotoxin unit

EU

standard unit of endotoxin activity

Note 1 to entry: The endotoxin unit was defined by the World Health Organization (WHO) Expert Committee on Biological Standardization (ECBS) in 1996, relative to the activity of 0,1 ng of WHO reference standard endotoxin (RSE) from *Escherichia coli* 0113:HK10:K(-) or 10 EU/ng

Note 2 to entry: EU is equal to international unit (IU) of endotoxin.

[SOURCE : ISO 29701:2010, definition 2.4]

3 Abbreviations:

LPS = Lipopolysaccharide
TLR4 = Toll-like-receptor 4
FDA = Food and Drug Administration
EMA = European Medicines Agency
LAL = Limulus Amoebocyte Lysate
RPT = rabbit pyrogen test
pNA = p-nitroaniline
EU/mL = endotoxin unit/mL

4 Principles of the Method:

Different assays to detect endotoxins have been described such as the rabbit pyrogen test (RPT) and the Limulus amoebocyte lysate (LAL) assay. These are the most commonly used endotoxin detection methods that are approved by the FDA and European Medicines Agency (EMA), and also accepted by almost any other country. Due to the high cost and long execution time of the assay in combination with the need of using animals, RPT is now mainly applied in combination with the

LAL test for analyzing parenteral drugs during the earlier development phase in biomedical research. For most other research fields, the most often used endotoxin detection method applied is the LAL assay since it represents a fast, sensitive and reasonably specific test method. Therefore, endotoxin contamination of the materials used by the PATROLS WP partners will be assessed following the Limulus Amoebocyte Lysate (LAL) assay. The LAL assay is originally based on the blood cell extract of the horseshoe crab (a marine arachnid), for LPS endotoxin testing. There are three major kinds of basic LAL tests: gel-clot, turbidimetric and chromogenic. Dobrovolskaia et al. have declared that none of the currently available LAL formats is optimal for endotoxin assessment for nanomaterial testing and suggested that at least two LAL formats with different endpoints/readouts should be used [9].

The gel-clot assay is the simplest LAL test and is used to detect the presence or absence of endotoxin by either forming a detectable gel-clot or not. The assay is based on the initiation of a series of enzymatic reactions after encountering with the endotoxin. The activation of this pathway results in the production of at least three serine protease zymogens: Factor C, Factor B, and a proclotting enzyme. These enzymes alter the amoebocyte coagulogen present in LAL to form a detectable gel-clot. However, the test is rather time consuming and provides no further information such as then concentration of the endotoxin. It can be used in a qualitative manner but not in a quantitative. In addition it has been shown that the gel clot LAL assay is not accurate for testing endotoxin contamination for different clinical-grade particles such as silica, silver, titanium dioxide, calcium carbonate and others (REF).

The turbidimetric assay is a technique that uses the change in gel turbidity to detect the activation of LAL reagent induced by endotoxin. The cleavage products coalesce as a result of ionic interactions that occur after the cleavage and cause the reaction mixture to become turbid. The turbidimetric method is sensitive to suspended or turbid materials and does often result in false positive results.

The chromogenic test is an optical analysis method that allows for qualitative as well as quantitative measurement of endotoxin through color changes. In the presence of endotoxin, the components of LAL are activated in a proteolytic cascade

that results in the cleavage of a colorless artificial peptide substrate present in Pyrochrome LAL. Proteolytic cleavage of the substrate liberates p-nitroaniline (pNA), which is yellow and has an absorbance of 405 nm. The degree and rate at which light is absorbed is directly proportional to the amount of endotoxin within the sample allowing quantitative data analysis. In addition, the chromogenic LAL assay showed higher sensitivity compared to the gel clot assay.

Out of the three different assays, the chromogenic testing system shows the easiest and fastest handling, higher sensitivity as well as qualitative and quantitative data outcome. For those reasons, the chromogenic endotoxin testing assays are recommended for the testing of the PATROLS nanomaterials.

5 Description of the Method:

5.1 Pierce LAL Chromogenic Endotoxin Testing

The endotoxin concentration in a sample is measured using the Pierce LAL Chromogenic Endotoxin Quantitation Kit via a chromogenic signal generated in the presence of endotoxins. Samples will be measured on a microplate absorbance reader at 405nm. A standard curve will be created using the *E. coli* endotoxin to calculate endotoxin levels as low as 0.1 EU/mL, where one endotoxin unit/mL (EU/mL) equals approximately 0.1ng endotoxin/mL of solution. Protein and antibody samples can be assayed in about 30 minutes.

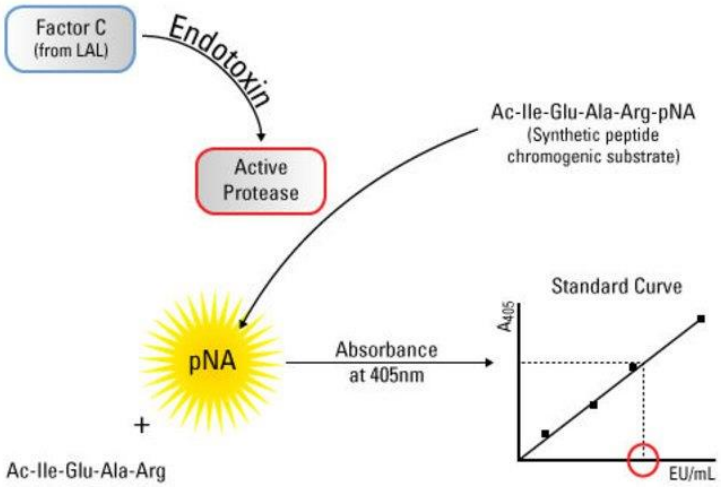

Figure 1: LAL Chromogenic Endotoxin Quantitation Kit reaction scheme. A small volume of the sample (10µL) is combined with the *Limulus* Amebocyte Lysate, and endotoxins in the sample activate the proteolytic activity of Factor C. When the chromogenic substrate is added, the activated protease catalyzes the cleavage of p-nitroalinine (pNA), resulting in yellow color that can be quantitated by measuring the absorbance at 405nm (A405) and extrapolating against a standard curve.

5.2 Endosafe® negxen-PTS™ Chromogenic Endotoxin Testing

The Endosafe® negxen-PTS™ is a rapid, point-of-use handheld spectrophotometer that utilizes disposable cartridges for accurate real-time endotoxin testing. The user simply pipettes 25µl of a sample into each of the four sample reservoirs of the cartridge. The reader draws and mixes the sample with the LAL reagent in the sample channels in addition to the LAL reagent plus positive control. The sample is combined with the chromogenic substrate and then incubated. After mixing, the optical density of the wells is measured and analyzed against an internally archived standard curve. By design, the cartridge technology automatically performs a duplicate sample/duplicate positive control LAL test.

Figure 2: Portable Endotoxin Testing System

5.3 Materials and Instruments:

Pierce LAL Chromogenic Endotoxin Quantitation Kit:

- Disposable endotoxin-free glass tubes or 1.5mL microcentrifuge tubes, pipette tips, 96-well microplates
- Heating block at 37°C ± 1°C
- Pipettor
- Microplate reader
- 25% acetic acid (Stop Reagent)
- LPS

Endosafe® negxen-PTS™ device:

- PTSTM Cartridges
- Endotoxin free reagent water
- Endoxotin free Dilution Tube
- Pipettor

5.4 Procedure:

Pierce LAL Chromogenic Endotoxin Testing:

1. Pre-equilibrate the microplate in a heating block for 10 minutes at 37°C ± 1° C
2. Incubate particles in endotoxin free water for 1h at 37° C
3. Spin down and collect supernatant (sample)
4. Dispense 50µL of each standard or unknown sample replicate into the appropriate microplate well. As a positive control LPS should be used.
5. At time T=0, add 50µL of LAL reagent to each well and incubate for 10min
6. After exactly T=10 minutes, add 100µL of Chromogenic Substrate solution (prewarmed to 37°C±1°C) to each well. Incubate the plate at 37° C ± 1°C for 6 minutes.

7. At T=16 minutes, add 100µL of Stop Reagent (25% acetic acid)
8. Measure the absorbance at 405 – 410 nm on a plate reader
9. Subtract the average absorbance of the blank replicates from the average absorbance of all individual standards and unknown sample replicates to calculate mean Δ absorbance
10. Use the formulated standard curve (linear regression) to determine the endotoxin concentration of each unknown sample

Endosafe® negxen-PTS™ Kinetic Chromogenic Endotoxin Testing:

1. Simply pipette 25µl of a sample into each of the four sample reservoirs of the cartridge

5.4.1 Testing for nanomaterial interference:

Depending on the physico-chemical characteristics such as the optical density, the materials could interfere with the LAL readouts. In order to eliminate the interference, dilution of the sample is recommended (REF). If the test sample endotoxin concentration is >1.0EU/mL, dilute the sample five-fold in endotoxin-free water. Re-test.

Assay inhibition occurs when substances in the test sample interfere with the LAL reaction. In the chromogenic assay, this inhibition results in a lower final absorbance, indicating lower levels of endotoxin than what may be present in the test sample. Determine the lack of product inhibition for each sample undiluted or at an appropriate dilution. To verify the lack of product inhibition, spike an aliquot or dilution of a test sample with a known amount of endotoxin (e.g., 0.5 EU/mL). Assay the spiked sample and the unspiked samples to determine the respective endotoxin concentrations. The difference between the two calculated endotoxin values should equal the known concentration of the spike ±25%.

6 References

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All references used within the main text of the SOP should be provided at this time. Please only use the Harvard style for citation of references.