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Practical Guide for Detection of Endotoxin in Nanoformulations using Limulus Amoebocyte Lysate (LAL) assays

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Cover Letter

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September 14, 2018

Nandita Singh, Ph.D.
Senior Science Editor

Dear Dr. Singh,

Thank you for the opportunity to revise the manuscript. With this letter, I am submitting the revised version of the paper entitled "Practical Guide for Detection of Endotoxin in Nanoformulations using Limulus Amoebocyte Lysate (LAL) assays" for your consideration for publication in the JoVE. The manuscript was revised according to the editorial and reviewers' comments. I am also submitting the detailed point-by-point responses to these comments. The edited text and responses to reviewers are highlighted in red font. The yellow highlight is used in the manuscript to identify the text which will be shown in the video portion of the paper.

By signing below, I certify that the data presented in the manuscript represent the original work not published elsewhere and has not been submitted for consideration to other journals. I also confirm that the authors of this manuscript do not have a financial conflict of interest related to this study.

Please do not hesitate to contact me should you have any questions about this submission.
Thank you for your time and consideration.
Sincerely,

Marina A. Dobrovolskaia, Ph.D.

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Frederick National Laboratory for Cancer Research

TITLE:

Detection of Endotoxin in Nano-formulations using Limulus Amoebocyte Lysate (LAL) assays

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KEYWORDS:

Endotoxin, nanoparticles, LAL assay, interference, spike recovery, pyrogenicity, contamination

SHORT ABSTRACT:

Detection of endotoxins in engineered nanomaterials represents one of the grand challenges in the field of nanomedicine. Here, we present a case study that describes the framework composed of three different LAL formats to estimate potential endotoxin contamination in nanoparticles.

LONG ABSTRACT:

When present in pharmaceutical products, a Gram-negative bacterial cell wall component endotoxin (often also called lipopolysaccharide) can cause inflammation, fever, hypo- or hypertension, and, in extreme cases, can lead to tissue and organ damage that may become fatal. The amounts of endotoxin in pharmaceutical products, therefore, are strictly regulated. Among the methods available for endotoxin detection and quantification, the Limulus Amoebocyte Lysate (LAL) assay is commonly used worldwide. While any pharmaceutical product can interfere with the LAL assay, nano-formulations represent a particular challenge due to their complexity. The purpose of this paper is to provide a practical guide to researchers inexperienced in estimating endotoxins in engineered nanomaterials and nanoparticle-formulated drugs. Herein, practical recommendations for performing three LAL formats including turbidity, chromogenic and gel-clot assays are discussed. These assays can be used to determine endotoxin contamination in nanotechnology-based drug products, vaccines, and adjuvants.

INTRODUCTION:

An endotoxin is a building block of the Gram-negative bacterial cell wall[1,2]. It can activate the immune cells at very low (picogram) concentrations[1,2]. The proinflammatory mediators (cytokines, leukotrienes, eicosanoids, *etc.*) produced by the cells in response to an endotoxin are responsible for fever, hypotension, hypertension, and more severe health problems including multiple organ failure[1-3]. The severity of the immune-mediated side-effects triggered by the

endotoxin depends on its potency determined by the endotoxin composition and structure and measured in international endotoxin units (IUs or EUs)[3]. The number of these units per kilogram of body weight is used to set a threshold pyrogenic dose of endotoxin. This dose is 5 EU/kg for drug products administered *via* all routes but the intrathecal route. Drugs dosed per square meter of body surface, intraocular fluids, radiopharmaceuticals, and products administered *via* intrathecal route have a different threshold pyrogenic dose, which is 100 EU/m^2, 0.2 EU/mL, 175 EU/V (where V is the volume of the product intended for administration), and 0.2 EU/kg, respectively[4]. More details about the threshold pyrogenic dose for various drug products and devices are provided and discussed elsewhere[4-6].

Animals vary widely in their sensitivity to endotoxin-mediated reactions. Humans, non-human primates, and rabbits are among the species most extremely sensitive to endotoxins[3]. To avoid endotoxin-mediated side effects in patients and prevent inaccurate conclusions of preclinical toxicity and efficacy studies, it is essential to accurately detect and quantify endotoxins in both clinical and pre-clinical grade formulations. Several currently available methods can achieve this task. One of them is the Limulus Amoebocyte Lysate (LAL) assay, which is commonly used worldwide to screen biomedical products for the potential endotoxin contamination as well as to detect bacterial infections[7-9]. The lysate is prepared from amoebocytes, the cells present in the blood of horseshoe crabs *Limulus polyphemus* residing in the east shore of the continent of North America[7]. Interestingly, there are a few different species of horseshoe crabs (*Tachypleus gigas* and *Tachypleus tridentatus)* in Asia[10]. The Tachypleus Amoebocyte Lysate (TAL) is used in several Asian countries for the detection of endotoxin similar to how the LAL is used in other cuntries[10]. The lysates (LAL and TAL) contain a group of proteins that upon activation confer protease activity. One of these proteins, the so-called Factor C is activated upon contact with endotoxin. Activated Factor C cleaves Factor B, which in turn also becomes a protease and cleaves a pro-clotting enzyme to produce a clotting enzyme. The result of this chain of reactions is the formation of a gel, an increase in the sample turbidity and, in the presence of a chromogenic substrate, the appearance of a colored product, which serve as a foundation for gel-clot, turbidity, and chromogenic assays, respectively. While there is no mandatory LAL format, the US Food and Drug Administration (FDA) explains in the guidance for industry document, that in case of discrepancy in the test results between different LAL formats, the decision is made based on the gel-clot assay[5].

Many commonly used laboratory chemicals (*e.g.*, EDTA) and known drug products (*e.g.,* penicillin) interfere with LAL assays[11]. The interference is usually identified by assessing the recovery of the endotoxin standard spiked at a known concentration into a solution containing the test material. If the spike recovery is less than 50% or more than 200%, then the result of the LAL assay for the given test material is invalid due to the inhibition or enhancement, respectively[4]. Nanotechnology-based formulations are often complex and interfere with the LAL through a variety of mechanisms[12-14]. Many approaches have been described to overcome the interference: sample reconstitution in specific buffers and surfactants, protein inactivation by heating, destruction of lipid-based hollow materials by heating and supplementing the sample with excess divalent cations[5,12-15]. Alternative methods for situations when LAL interference cannot be

overcome have also been described: ELISA, a HEK-TLR4 reporter cell line assay, and mass spectrometry[16-19].

Herein, experimental procedures for conducting gel-clot, turbidity, and chromogenic LAL assays are described. These assays are also available on the Nanotechnology Characterization Lab (NCL) website[20] in protocols STE1.2 (turbidity LAL), STE1.3 (gel-clot LAL) and STE1.4 (chromogenic LAL). It is recommended to conduct at least two different formats to characterize the same nano-formulation. When results of the turbidity and chromogenic LAL disagree, the gel-clot results are considered[5]. When results of two LAL formats disagree, additional studies using either monocyte activation test (MAT) or rabbit pyrogen test (RPT) to verify LAL findings are conducted[21]. It is important to note that each method used for endotoxin detection and pyrogenicity assessment has advantages and limitations[21-24]. Recognizing limitations of the procedure used to characterize a given nanotechnology formulation is essential to obtain scientific justification for the use of the procedure optimal for that nano-formulation.

In this study, PEGylated liposomal doxorubicin was used as a model nanoparticle formulation. This formulation has been approved by the US FDA in 1995 and used for treating cancer patients worldwide[25].

PROTOCOL:

1. Preparation of Nanoparticle Samples

1.1. Prepare the study sample in LAL grade water.

1.2. If the sample pH is outside of the 6-8 range, adjust the pH by using pyrogen-free sodium hydroxide or hydrochloric acid.

1.3. Using LAL grade water prepare several dilutions of the study sample. Make sure that the highest dilution does not exceed maximum valid dilution (MVD). Refer to the discussion section for details about MVD estimation.

2. Preparation of Reagents Common Between LAL Formats

2.1. Dilute concentrated sodium hydroxide stock using pyrogen-free LAL reagent water to prepare a working solution at a concentration of 0.1 N.

2.2. Dilute concentrated hydrochloric acid stock using pyrogen-free LAL reagent water and prepare a working solution at a final concentration of 0.1 N.

2.3. Preparation of the Control Standard Endotoxin (CSE)

2.3.1. Reconstitute the CSE according to the certificate of analysis supplied by the manufacturer.

NOTE: Refer to the discussion section for important notes regarding the information provided in the certificate. Refer to the **Table of Materials** for the details regarding catalog number and application of a given CSE formulation in different LAL formats.

2.4. Preparation of the LAL Reagent

2.4.1. Reconstitute the LAL reagent according to the certificate of analysis provided by the manufacturer.

NOTE: Refer to the discussion section for important details regarding LAL reagent preparation. Refer to the **Table of Materials** for the details regarding catalog number and application of a given LAL reagent formulation in different LAL format.

3. Turbidity LAL Assay

3.1. Preparation of the Calibration Standards

3.1.1. Using 900 µL of LAL grade water and 100 µL of CSE, prepare as many intermediate dilutions as needed to enable the preparation of a calibration standard with a concentration of 1 EU/mL.

3.1.2. Using 900 µL of LAL-grade water and 100 µL of the 1 EU/mL calibration standard, prepare a second calibration standard at a concentration of 0.1 EU/mL.

3.1.3. Repeat the serial 10-fold dilution as described above to prepare two lower calibration standards. Verify that four calibration standards ranging from 0.001 to 1 EU/mL have been prepared.

3.2. Preparation of the Quality Controls

3.2.1. Prepare a 0.05 EU/mL quality control by combining 50 µL of the 1 EU/mL CSE solution with 950 µL of LAL-grade water.

NOTE: Refer to the discussion section for details regarding control preparation.

3.3. Preparation of Inhibition/Enhancement (IEC) Controls

3.3.1) Prepare 0.05 EU/mL IEC by combining 50 µL of the 1 EU/mL CSE solution with 950 µL of the test nanomaterial.

NOTE: Refer to the discussion section for additional details.

3.4. Experimental procedure

3.4.1. Allow the instrument to warm up by turning it on approximately 30 min in advance. Set-up the detection wavelength to 660 nm as this is appropriate for the turbidity LAL.

3.4.2. Open the software (**Table of Materials**) by clicking on the corresponding icon on a computer screen. Sign in by typing the username and password.

3.4.3. Select **Collect data** on the software home screen. Enter the test ID and data group information into the corresponding space in the **General** tab on the home screen.

3.4.4. Click the **Hardware** tab. Choose the instrument type from a dropdown menu. Choose the instrument.

3.4.5. Verify that a serial number, system ID and serial port information appear on the screen. Click **OK**. Click **OK** one more time to confirm.

3.4.6. Enter the sample ID in the same order the sample is tested. Use default buttons to enter the negative control, standard curve and test samples.

3.4.7. Prepare duplicate tubes for each sample and add 200 µL (test ration 4:1) or 100 µL (test ratio 1:1) of negative control (water), calibration standards, quality control, IEC and test nanoparticles into pre-labeled glass tubes.

3.4.8. Add 50 µL (test ratio 4:1) or 100 µL (test ratio 1:1) of LAL reagent to first test vial, vortex it briefly, and insert into test slot in the instrument carousel. If the 1:1 ratio is used, the volume of the LAL reagent is 100 µL.

3.4.9. Repeat the procedure described above for other samples. Process samples one at a time.

NOTE: Refer to the discussion section for more details.

4. Chromogenic LAL

4.1. Preparation of Calibration Standards

4.1.1. Using 900 µL of LAL grade water and 100 µL of CSE, prepare as many intermediate dilutions as needed to enable the preparation of a calibration standard with a concentration of 1 EU/mL.

4.1.2. Using 900 µL LAL-grade water and 100 µL of the 1 EU/mL calibration standard, prepare a second calibration standard at a concentration of 0.1 EU/mL.

4.1.3. Repeat the serial 10-fold dilution as described above to prepare two lower calibration standards. Verify that four calibration standards ranging from 0.001 to 1 EU/mL have been prepared.

4.2. Preparation of Quality Controls.

4.2.1. Prepare a 0.05 EU/mL quality control by combining 50 µL of the 1 EU/mL CSE solution with 950 µL of LAL-grade water.

NOTE: Refer to the discussion section for details regarding control preparation.

4.3. Preparation of Inhibition/Enhancement (IEC) Controls

4.3.1. Prepare 0.05 EU/mL IEC by combining 50 µL of the 1 EU/mL CSE solution with 950 µL of test nanomaterial.

NOTE: Refer to the discussion section for additional details.

4.4. Experimental procedure

4.4.1. Allow the instrument to warm up by turning it on approximately 30 min in advance. Set-up the detection wavelength to 405 nm as this is appropriate for the turbidity LAL.

4.4.2. Open the software by clicking on the corresponding icon on a computer screen. Sign in by typing the username and password.

4.4.3. Select **Collect data** on the software home screen. Enter test ID and data group information into corresponding space in the **General** tab on the home screen.

4.4.4. Click the **Hardware** tab. Choose the instrument type from a dropdown menu. Choose the instrument.

4.4.5. Verify that a serial number, system ID and serial port information appear on the screen. Click **OK**. Click **OK** one more time to confirm.

4.4.6. Enter the sample ID in the same order the sample is tested. Use default buttons to enter negative control, standard curve and test samples.

4.4.7. Prepare duplicate tubes for each sample and add 200 µL (test ration 4:1) or 100 µL (test ratio 1:1) of negative control (water), calibration standards, quality control, IEC and test nanoparticles into pre-labeled glass tubes.

4.4.8. Add 50 µL (test ratio 4:1) or 100 µL (test ratio 1:1) of LAL reagent to first test vial, vortex it briefly, and insert into test slot in the instrument carousel. If the 1:1 ratio is used, the volume of the LAL reagent is 100 µL.

4.4.9. Repeat the procedure described above for other samples. Process samples one at a time.

5. Gel-Clot LAL

NOTE: This assay identifies the presence of endotoxins in the sample based on the visual observation and detection of a clot in the reaction tube. The experimental steps are described below. Use a bench sheet to record the results. This bench sheet is not mandatory, and other ways of recording the assay results are also acceptable. An example of such a bench sheet is provided in supplementary materials for the convenience of a reader. Lambda (λ) is the sensitivity of the gel-clot assay and is 0.03 EU/mL.

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

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

5.3. Prepare CSE such that the final concentration is equal to 4λ.

5.4. Combine 100 µL of the standard mentioned above with 100 µL of water or test sample to achieve the final concentration of CSE of 2λ.

5.5. Make sure that the temperature in the water bath is 37 °C.

5.6. Add 100 µL of lysate per test tube, vortex briefly and place the rack with all the tubes into the water bath for 1 h.

5.7. Invert the tube with a smooth motion.

5.8. Manually record results using "**+**" (firm clot) or "**-**" (no clot or loose clot) on the bench sheet.

5.9. Proceed with the analysis according to the USP BET 85[4];use the bench sheet as supporting material

REPRESENTATIVE RESULTS:

The example of data generated after testing this formulation in LAL assays is shown in **Table 1**. PEGylated liposomal doxorubicin interfered with chromogenic LAL at dilution 5. However, this interference was overcome by greater dilutions. Spike recovery was between 50 and 200% when this formulation was tested at dilutions 50 and 500 in turbidity and chromogenic LAL, as well as at dilution 5 in turbidity LAL. When adjusted by the dilution factor, the results were consistent between dilutions in both assays. Moreover, the results were consistent between the three assay formats.

FIGURE AND TABLE LEGEND:

Table 1: Detection of endotoxin in PEGylated liposomal doxorubicin using LAL assays. PEGylated liposomal doxorubicin was tested using turbidity, chromogenic and gel-clot LAL assays. Sample interference was estimated using IECs. Spike recovery is shown in parenthesis. Values between 50 and 200% are considered acceptable according to the USP BET 85 standard for endotoxin detection[4]. * and ** the test results of this sample were obtained at dilutions 100 and 200, respectively. The gel-clot assay dilutions are different from those used in chromogenic and turbidity LAL because sensitivity and maximum valid dilution of this assay are lower.

DISCUSSION:

The information provided in this protocol has been described before[15,26] and relies on several regulatory documents published by the US Food and Drug Administration (US FDA or FDA) and United States Pharmacopoeia (USP)[4-6,27], and is also available on the NCL website[20] in protocols STE1.2 (turbidity LAL), STE1.3 (gel-clot LAL) and STE1.4 (chromogenic LAL).

Test-nanomaterials are prepared either in LAL reagent water or sterile, pyrogen-free PBS. The pH of the study sample is important because low (< 6) and high (> 8) pH will interfere with the optimal performance of the lysate. If the pH of the test-nanoparticle is outside of the 6-8 range, it can be adjusted using pyrogen-free sodium hydroxide or hydrochloric acid. When such adjustment is performed, it is vital to avoid sample contamination from microelectrode. Therefore, to perform this procedure, a small aliquot of the sample is removed into a separate tube and used to measure the pH.

When the sample is prepared in PBS or another buffer, the blank buffer is also included in this test. The concentration of each nanomaterial is case-specific. The test is used to estimate the amount of contaminating endotoxin per milligram of the active pharmaceutical ingredient (API). However, depending on the type of nano-formulation, the concentration can also be estimated in mg of the total formulation or total element (*e.g.*, gold, silver or iron for gold, silver, and iron oxide nanoparticles, respectively). The sample is tested from the stock using several dilutions not exceeding the MVD. All dilutions of the test-nanoparticles are prepared using LAL-grade water.

Three parameters are used to calculate the MVD. They are the endotoxin limit (EL), the sample concentration, and the assay sensitivity (λ)[4]. Use the following formula to calculate endotoxin limit (EL): EL=K/M, where K is a threshold pyrogen dose (*e.g.*, 5 EU/kg as discussed in the introduction) and M is the maximum dose of the test nanomaterial intended for administration per kilogram of body weight in a single hour[4]. As discussed above, the estimation of EL for nanomaterials used as radiopharmaceutical or as medical device relies on a threshold pyrogenic dose or formula, which is different from 5/M. These details are mentioned in the introduction and could be further reviewed in the USP and US FDA guidelines[4-6]. The recommended endotoxin limit for ophthalmic solutions and intraocular devices is provided in the US FDA guideline for these products[6]. When an animal model (*e.g.*, mouse) was used to establish the dose of nanoformulation, this information is used to estimate the so-called human equivalent dose (HED). The HED is calculated by dividing the animal dose by a conversion factor, which is specific to each animal species. For example, the conversion factor is 12.3, 6.2 and 3.1 for the mouse, the rat, and the rabbit, respectively[27]. The conversion procedure and rationale for using it are

described in detail in the US FDA guideline[27]. Cancer therapeutics are often dosed per surface body area expressed in mg/m^2. One can either follow the USP guideline to calculate EL for drugs dosed in milligram per square meter or convert this dose to mg/kg range. The dose in mg/m^2 can be adjusted to the dose in mg/kg using a species-specific conversion factor[27]. For an adult human, it is 37 and indicated as km to refer to the mass constant[27]. The km factor has units of kg/m^2; it is equal to the body weight in kilograms (kg) divided by the surface area in square meters (m^2)[27]. For example, a human dose or HED of 74 mg/m^2 corresponds to 2 mg/kg or 74/37. To determine MVD, use the following formula, which is also available from the USP BET 85 standard: MVD= (EL x sample concentration)/λ)^4. In a hypothetical scenario, a nanoparticle sample concentration is 10 mg/mL and its maximum dose in the mouse is 615 mg/kg. In this case the HED is 615/12.3=50 mg/kg; EL for all routes except intrathecal is 0.1 EU/mg (5 EU/kg/50 mg/kg) and MVD is 1000 ((0.1 EU/mg x 10 mg/mL)/0.001 EU/mL).

Often, when one needs to evaluate endotoxin contamination in a research grade nanomaterial, the dose information is not available. There is no harmonized procedure for how to estimate the MVD and EL for these materials. This case study performs the test directly from stock (commonly the concentration of this solution is 1 mg/mL) and at several dilutions, usually 5-, 50-, 500- and 5000- folds. When the dose, the route of administration, the sample concentration and ithe nfusion time for the given test nanomaterial change, the EL and MVD also change. Therefore, one has to evaluate the information and perform estimation of EL and MVD in the context of other parameters related to the amount, time, intended route of administration and concentration of the given formulation.

It is important to note that the catalog number and the product specifications (*e.g.*, potency, amounts per vial) of the CSE are different for different LAL formats. The CSE used in turbidity LAL can also be used in a gel-clot LAL. However, the CSE for a chromogenic LAL is specific to this assay format. The instructions provided below are general and applicable to the CSE used in all assay formats. The CSE is an *E. coli* lipopolysaccharide (LPS) supplied as a lyophilized powder. This standard is certified by the manufacturer against a Reference Standard Endotoxin (RSE) with a known potency. The contents of the vial containing CSE should be reconstituted with ~3.2 - 5.0 mL of pyrogen-free LAL reagent water. In addition to the difference between CSE used for different assay formats, the volume of reconstitution is also specific to each lot of this standard. Therefore, one has to calculate the final concentration of the stock CSE solution for each lot of the standard. The calculation is performed based on the potency of the standard and the amount contained in each vial. The certificate of analysis specific for each lot of endotoxin standard contains the information about the potency and amount per vial. One has to rigorously vortex the standard for 30-60 seconds, both during the reconstitution and during the use. It is also recommended to perform the reconstitution using 5-10 min settling times and over a 30-60 min time frame to ensure that all lyophilized material goes into solution. Before using in the assay, equilibrate the stock CSE to room temperature. After the reconstitution, the CSE stock can be refrigerated for storage and is stable for four weeks.

Similar to the CSE, the LAL reagent is also specific for each format. The instructions provided below are general and applicable to all LAL assay formats. The LAL reagent is provided as a

lyophilized powder. The manufacturer's recommendations are followed to reconstitute each vial. Either LAL grade water or buffer inhibiting beta-glucans can be used. The buffer inhibiting beta-glucans is preferred in this case-study because it allows excluding the interference from beta-glucans. This false-positive interference is very common in nanomaterials because cellulose-acetate filters are commonly used during nanomaterial synthesis and serve as a source of glucan contamination[15]. Most vials will require reconstitution to a final volume of 5 mL. Based on over 10 years of the authors' experience with this assay, it has been noticed that the inclusion of this buffer slows down the reaction and may require increasing the maximum onset time in the instrument settings to allow the lowest calibrator at a concentration of 0.001 EU/mL to develop.

To ensure accurate dilution during the preparation of standards, it is recommended to use 10-fold dilution steps. Serial 10-fold dilutions can be prepared by spiking 100 µL of the stock or a standard with higher concentration into 900 µL of pyrogen-free water. Since the concentration of the CSE stock is usually high (~1000 EU/mL), preparation of intermediate dilutions with concentrations 100 and 10 EU/mL is recommended before preparing the assay calibrator with a concentration of 1 EU/mL. Two ratios between the test sample and lysate are described in this protocol. Even though both ratios are commonly used, the curve linearity is acceptable but not ideal when the 4:1 ratio and the buffer inhibiting beta-glucans (see the **Table of Materials**) are used.

The same rules as described above regarding the calibration standard preparation also apply to the preparation of quality controls (QC). These controls are used to verify that the assay is functioning correctly. It is prepared by spiking the known amount of CSE into the pyrogen-free water. The concentration of QC is usually chosen in the middle of the assay range. The range of the turbidity LAL assay is 0.001 to 1 EU/mL. Therefore, a QC at a concentration of 0.05 EU/mL is used. Other concentrations within the assay range can also be used in the preparation of the quality control.

The inhibition enhancement control (IEC) is also known as positive product control (PPC). It is prepared by spiking a known concentration of CSE into the test sample. The concentration of IEC (PPC) is usually chosen in the middle of the assay range. The range of the turbidity LAL assay is 0.001 to 1 EU/mL. Therefore, the IEC at a concentration of 0.05 EU/mL is used. To prepare this IEC one needs to combine 50 µL of the 1 EU/mL CSE solution with 950 µL of test-nanoparticle. The IEC is prepared for each dilution of a test-nanomaterial. For example, if a nano-formulation is tested at three dilutions (1:50, 1:500 and 1:5000), one has to prepare three IECs, one for each of these dilutions. Other concentrations within the assay range can also be used to prepare the IEC sample. This control is used to understand the validity of the test results for the given nano-formulation. According to the USP, the test-results are valid if the spike recovery of IEC (PPC) is between 50 and 200%[4]. Spike recovery less than 50% means that the test-nanomaterial inhibits endotoxin detection and, therefore, the test result underestimates endotoxin contamination and is invalid. Spike recovery over 200% suggests that the test-material enhances the assay result or contains a too high level of the contaminating endotoxin. In the case of enhancement, the assay result is also inaccurate. Examples of such interferences and some ways for overcoming them have been described earlier[12,15].

When performing turbidity and chromogenic assays, it is recommended to use a repeater pipette to add the LAL reagent to all samples. The average instrument operation time is 7200 seconds. However, the reaction can be faster or slower with certain lots of the lysate. In cases, when the reaction is slow, one may need to change the instrument settings to 9600s or longer. This change will allow the lowest calibrator to develop.

PEGylated liposomal doxorubicin is a formulation containing an active pharmaceutical ingredient (API) at a concentration of 2 mg/mL. It is used in the clinic as a dose of 50 mg/m^2. To convert this dose to mg/kg range, it is divided by factor 37[27]. The dose of PEGylated liposomal doxorubicin in mg/kg, therefore, is 1.35. To calculate EL, 5 (the threshold pyrogen dose in EU/kg) is divided by 1.35 (the dose of Doxil in mg/kg) and obtain 3.7 EU/mg[4]. This number means that PEGylated liposomal doxorubicin at the dose of 1.35 mg/kg can be safely administered per single hour if endotoxin in the formulation does not exceed 3.7 EU/mg, where mg refers to the API.

Next, use this information to calculate the MVD for LAL assays. The sensitivity (lambda) of the turbidity, chromogenic and gel-clot assays presented in this study is 0.001, 0.001 and 0.03 EU/mL, respectively. Therefore, MVD is 7407 for turbidity and chromogenic assays ((5 EU/kg x 2 mg/mL)/0.001 EU/mL), and 247 for the gel-clot assay ((5 EU/kg x 2 mg/mL)/0.03 EU/mL).

Doxorubicin has a broad absorbance spectrum that overlaps with assay wavelength of the chromogenic LAL[28,29]. Moreover, most liposome formulations are turbid and appear milky. This inherent turbidity is the very common reason for the interference of liposomes with turbidity assay. In the case of PEGylated liposomal doxorubicin, the interference due to the turbidity was overcome by dilution five as evidenced by the spike recovery values between 50 and 200% (**Table 1**). However, at the same dilution doxorubicin concentration was still high enough to interfere with the chromogenic LAL (**Table 1**). Two subsequent dilutions (50 and 500) helped to overcome PEGylated liposomal doxorubicin interference with chromogenic LAL. Since the gel-clot assay is independent of turbidity and sample color, and the level of endotoxin in the formulation is within the gel-clot range, the PEGylated liposomal doxorubicin did not interfere with the gel-clot assay at all tested dilutions. In this case study, several dilutions of PEGylated liposomal doxorubicin were used for these assays. The dilutions 5, 50 and 500 for turbidity and chromogenic as well as 50, 100 and 200 for the gel-clot assay were chosen because they are within the MVD. The MVD of the gel-clot assay is lower than that of the turbidity and chromogenic LALs because the sensitivity of this assay is also lower. If the formulation interfered with the LAL at dilution 500 in turbidity and chromogenic assays, the analysis at higher dilutions (*e.g.,* 5000, 6000, 7000 or 7407) could be performed. Since the level of endotoxin obtained at dilution 50 in the gel-clot assay was below the EL, the analysis of this formulation at lower dilutions was not performed. However, in other cases, the testing could be continued at Dilutions 20, 10, 5 or 2 to identify the lowest not interfering dilution and understand whether the level of endotoxin in the formulation is below the EL.

It is important to mention that for the gel-clot assay it is essential to turn off the water circulation function in the water bath for the duration of this assay or use a water bath without such option

because water flow damages the clot and may affect the accuracy of the gel-clot LAL assay. It is not always possible to overcome nanoparticle interference with LAL assays by increasing the sample dilutions. Strategies and some approaches for overcoming various types of interference and examples of nanoparticles commonly representing a problem for LAL assays have been discussed by other groups and us elsewhere[12-15,30,31]. The analysis presented in this manuscript is based on a model formulation. The experience with other nanoformulations may be different.

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Table 1: Table 1 Detection of endotoxin in PEGylated liposomal doxorubicin using LAL assays. PEGylated liposomal doxorubicin (see the table of materials) was tested using turbidity, chromogenic and gel-clot LAL assays. Sample interference was estimated using IECs. Spike recovery is shown in parenthesis. Values between 50 and 200% are considered acceptable according to the USP BET 85 standard for endotoxin detection[4]. * and ** the test results of this sample were obtained at dilutions 100 and 200, respectively. The gel-clot assay dilutions are different from those used in chromogenic and turbidity LAL because sensitivity and maximum valid dilution of this assay are lower.

Test sample	Turbidity LAL, EU/mg (spike recovery,%)	Chromogenic LAL, EU/mg, (spike recovery,%)	Gel-clot LAL, EU/mg (test valid, Yes or No)
PEGylated liposomal doxorubicin (see the table of materials) Dilution 5 Dilution 50 Dilution 500	0.01 (141) < 0.025 (187) < 0.25 (182)	interference 0.029 (82) < 0.25 (86)	< 0.75 (Y) < 1.5 (Y)* < 3 (Y)**

Table 1

Test sample	Turbidity LAL, EU/mg (spike recovery,%)	Chromogenic LAL, EU/mg, (spike recovery,%)
PEGylated liposomal doxorubicin (see the Table of Materials)		
Dilution 5	0.01 (141)	interference
Dilution 50	< 0.025 (187)	0.029 (82)
Dilution 500	< 0.25 (182)	< 0.25 (86)

Gel-clot LAL, EU/mg (test valid, Yes or No)
< 0.75 (Y) < 1.5 (Y)* < 3 (Y)**

Table of Materials

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Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Turbidity LAL Assay			
Sodium Hydroxide	Sigma	S2770	When needed, it is used to adjust sample pH to be between 6-8
Hydrochloric acid	Sigma	H9892	When needed, it is used to adjust sample pH to be between 6-8
LAL Reagent	Associates of Cape Cod	T0051	This reagent can be used with turbidity assay only
Control Endotoxin Standard	Associates of Cape Cod	E0005	This reagent can be used with turbidity and gel-clot assays
LAL grade water	Associates of Cape Cod	WP0501	This reagent can be used with any LAL format
Glucashield Buffer	Associates of Cape Cod	GB051-25	Used to prevent false-positive response from beta-glucans
Disposable endotoxin-free glass dilution tubes 12 x 75 mm	Associates of Cape Cod	TB240	These tubes can be used with all three assays
Disposable endotoxin-free glass reaction tubes 8 x 75 mm	Associates of Cape Cod	TK100	These tubes can be used with turbidity and chromogenic assays
Pyrogen-free tips with volumes 0.25 and 1.0 mL	RAININ	PPT25, PPT10	Tips and pipettes may adsorb endotoxin and release leachables which interfere with LAL assay. These RAININ tips are used because their optimal performance in the LAL assay was verified and confirmed
Pyrogen-free microcentrifuge tubes, 2.0 mL	Eppendorf	22600044	Other equivalent supplies can be used
Pyrogen-fee combitips, 5mL	Eppendorf	30089669	Other equivalent supplies can be used
Repeat pipettor	Eppendorf	4982000020	Other equivalent supplies can be used
Microcetrifuge	any brand		Any brand can be used
Refrigerator, 2-8 C	any brand		Any brand can be used
Vortex	any brand		Any brand can be used
Freezer, -20 C	any brand		Any brand can be used
Pyros Kinetix or Pyros Kinetix Flex reader	Associates of Cape Cod	PKF96	Other instruments can be used. However, LAL reagents and endotoxin standards used in this assay may require optimization. When other instrumentation is used, please refer to the instrument and LAL kit manufacturers for instructions
Chromogenic LAL Assay			
Pyrochrome LAL Reagent	Associates of Cape Cod	CG1500-5	This reagent is specific to the Chromogenic Assay
Control Endotoxin Standard	Associates of Cape Cod	EC010	This standard is different than that used for turbidity and gel-clot LALs; it is optimized for optimal performance in the chromogenic assay
Sodium Hydroxide	Sigma	S2770	When needed, it is used to adjust sample pH to be between 6-8
Hydrochloric acid	Sigma	H9892	When needed, it is used to adjust sample pH to be between 6-8
LAL grade water	Associates of Cape Cod	WP0501	This reagent can be used with any LAL format
Glucashield Buffer	Associates of Cape Cod	GB051-25	Used to prevent false-positive response from beta-glucans
Disposable endotoxin-free glass dilution tubes 12 x 75 mm	Associates of Cape Cod	TB240	These tubes can be used with all three assays
Disposable endotoxin-free glass reaction tubes 8 x 75 mm	Associates of Cape Cod	TK100	These tubes can be used with turbidity and chromogenic assays
Pyrogen-free tips with volumes 0.25 and 1.0 ml	RAININ	PPT25, PPT10	Tips and pipettes may adsorb endotoxin and release leachables which interfere with LAL assay. These RAININ tips are used because their optimal performance in the LAL assay was verified and confirmed
Pyrogen-free microcentrifuge tubes, 2.0 mL	Eppendorf	22600044	Other equivalent supplies can be used
Pyrogen-fee combitips, 5mL	Eppendorf	30089669	Other equivalent supplies can be used
Repeat pipettor	Eppendorf	4982000020	Other equivalent supplies can be used
Microcetrifuge	any brand		Any brand can be used
Refrigerator, 2-8 C	any brand		Any brand can be used
Vortex	any brand		Any brand can be used
Freezer, -20 C	any brand		Any brand can be used
Pyros Kinetix or Pyros Kinetix Flex reader	Associates of Cape Cod	PKF96	Other instruments can be used. However, LAL reagents and endotoxin standards used in this assay may require optimization. When other instrumentation is used, please refer to the instrument and LAL kit manufacturers for instructions
Gel-Clot LAL Assay			
LAL Reagent	Associates of Cape Cod	G5003	This reagent is specific to the gel-clot assay
Control Endotoxin Standard	Associates of Cape Cod	E0005	This reagent can be used with turbidity and gel-clot assays
Sodium Hydroxide	Sigma	S2770	When needed, it is used to adjust sample pH to be between 6-8

Hydrochloric acid	Sigma	H9892	When needed, it is used to adjust sample pH to be between 6-8
LAL grade water	Associates of Cape Cod	WP0501	This reagent can be used with any LAL format
Glucashield Buffer	Associates of Cape Cod	GB051-25	Used to prevent false-positive response from beta-glucans
Disposable endotoxin-free glass dilution tubes 12 x 75 mm	Associates of Cape Cod	TB240	These tubes can be used with all three assays
Disposable endotoxin-free glass reaction tubes 10 x 75 mm	Associates of Cape Cod	TS050	These tubes are for use with the gel-clot assay
Pyrogen-free tips with volumes 0.25 and 1 mL	RAININ	PPT25, PPT10	Tips and pipettes may adsorb endotoxin and release leachables which interfere with LAL assay. These RAININ tips are used because their optimal performance in the LAL assay was verified and confirmed
Pyrogen-free microcentrifuge tubes, 2.0 mL	Eppendorf	22600044	Other equivalent supplies can be used
Pyrogen-fee combitips, 5mL	Eppendorf	30089669	Other equivalent supplies can be used
Repeat pipettor	Eppendorf	4982000020	Other equivalent supplies can be used
Microcetrifuge	any brand		Any brand can be used
Refrigerator, 2-8 C	any brand		Any brand can be used
Vortex	any brand		Any brand can be used
Freezer, -20 C	any brand		Any brand can be used
Water bath, 37 C	any brand		Any brand can be used, however, it is important either to switch off water circulation or use non-circualting water bath because water flow will affect clot formation and lead to false-negative results

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