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

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1.1	01.10.2016	Chapter 7	Updated Acceptance criteria now in line with NCI-NCL acceptance criteria	Matthias Rösslein

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 turbidimetric Limulus Amebocyte Lysate (LAL) assay. Principle of the Method

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 photometrically with an automated microplate reader 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]. The method is based the initial reaction of the LAL with endotoxin. A LAL proenzyme is activated in the presence of endotoxin. As a result of the following cascade of enzyme activation steps coagulation is initiated and turbidity of the reaction mixture increases. The development of turbidity is measured using an automated plate reader and the time to reach a specific increment of turbidity (the onset time) is determined. Higher endotoxin concentrations give shorter onset times. Concentration of endotoxin in a sample is calculated from a standard curve prepared by the onset time of known concentration of endotoxin standard into LAL grade water. This method relies on Limulus Amebocyte Lysate PYROTELL®–T by Pyroquant Diagnostik GmbH, a subsidiary of Associates of Cape Cod, Inc. (ACC) [3]. Data analysis is performed using MARS software (BMG Labtech GmbH).

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.

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-T [3]. Use Pyrotell-T for in vitro diagnostic purposes only. Do not use it for the detection of endotoxemia [3].

4 Related Documents

Table 1:

Document ID	Document Title
NCL Method STE-1.2	**Detection and Quantification of Gram Negative Bacterial Endotoxin Contamination in Nanoparticle Formulations by Kinetic Turbidity 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 Reagent reservoirs (Lonza 00190035 or equivalent)

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 automated Microplate reader, temperature controlled 37°C, at 340 nm absorption (e. g. Novostar®, ClarioStar®, BMG Labtech GmbH)

5.2 Reagents

5.2.1 Test nanomaterial

5.2.2 LIMULUS AMEBOCYTE LYSATE PYROTELL®–T For The Detection And Quantification Of Gram Negative Bacterial Endotoxins (PYROQUANT DIAGNOSTIK, Associates of Cape Cod, Inc. (ACC))

5.2.3 Control Standard Endotoxin (CSE) (PYROQUANT DIAGNOSTIK GmbH, ACC)

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

5.2.5 LAL Reagent Water (PYROQUANT DIAGNOSTIK GmbH, ACC)

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

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

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-T 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-T LAL Reagent only immediately before use. Add the volume as indicated on the vial label, which usually is 5 mL, and is good to perform 48 single reactions. 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 extreme caution to avoid formation of air bubbles. Do not vortex the reconstituted lysate. Pipette with caution. Mix only by very gently swivel to avoid foaming. In case of bubbles allow to clear before use. Cover the vial with Parafilm M® when not in use.

Store the lyophilized Pyrotell-T 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 for the Kinetic Turbidimetric Microplate Method (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.

5.3.3 Preparation of Endotoxin calibration standards

If the Pyrotell-T LAL Endotoxin Detection System (Associates of Cape Cod, Inc.) is being used in a microplate reader the detection limit, and thus the lowest possible value of λ is 0.005 EU/mL [3]. Adjust the quantitative range of the assay and the sensitivity of an individual test defined by the lowest endotoxin concentration used to construct the standard curve.

Label disposable pyrogen-free glass dilution tubes for the endotoxin dilutions. Prepare a serious of endotoxin standard-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 may be prepared as described in the following table (alternative dilution schemes may be used):

Dilution scheme for preparation of a series of endotoxin standard dilutions

Sample	Nominal Concentration (EU/mL)	Preparation Procedure
Int. A *	50 *	100 µL Stock CSE + 1900 µL LAL reagent water
Cal. 1	5.0	100 µL of Int. A 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

* This is an example; dilution of the CSE Stock to make Int. A solutions depends on the concentration of CSE stock and is determined for each lot of CSE reagent, refer to the Custom Certificate Of Analysis. **Numbers shown in the table above are calculated based on a Stock concentration of 1000 EU/mL.**

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

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 should equal that of a standard dilution from the middle of the standard

curve and should be the same as used in the Quality Controls. For example, 25 µL of a 10 EU/mL Endotoxin standard dilution are added to 475 µL nanoparticle suspension of the sample, resulting in a spiked endotoxin concentration of 0.5 EU/mL. For the Quality control, the same amount of Endotoxin is diluted in to 475 µL LAL reagent water.

Dilution scheme: Preparation of Positive Product Controls (PPC)

Sample	Nominal Concentration (EU/mL)	Preparation Procedure	
Int. A **	100.0 *	100 µL Stock CSE	+ 900 µL LAL reagent water
Int. B **	10 *	100 µL of Int. A	+ 900 µL LAL reagent water
IEC	0.5	25 µL of Int. B	+ 475 µL nanoparticle suspension***

* Numbers shown in the table above are calculated based on a Stock concentration of 1000 EU/mL.

** Intermediate solutions A, B are prepared only to make control dilutions and are not used in assay.

*** The concentration of nanoparticles in IEC should be equal to one assayed for standard curve. You will need to prepare an IEC for each dilution of the nanomaterial assayed in this test.

Each sample should be vigorously vortexed for at least one minute prior to use. Transfer 100 µL of the IEC solution into the 96-well plate as directed by the assay template.

5.4.2 Quality Controls

Dilution scheme: Preparation of Quality Controls

Sample	Nominal Concentration (EU/mL)	Preparation Procedure	
Int. A **	100.0 *	100 µL Stock CSE	+ 900 µL LAL reagent water
Int. B **	10.0 *	100 µL of Int.A	+ 900 µL LAL reagent water
QC	0.5	25 µL of Int. B	+ 475 µL LAL reagent water

* Numbers shown in the table above are calculated based on a Stock concentration of 1000 EU/mL.

** Intermediate solutions A, B are prepared only to make control dilutions and are not used in assay.

*** The nominal concentration in QC should equal that of a standard from the middle of the standard

curve and should be the same as in IEC.

Each sample should be vigorously vortexed for at least one minute prior to use. Transfer 100 µL of the QC solution into the 96-well plate as directed by the assay template.

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. 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.

6.3 Flow chart

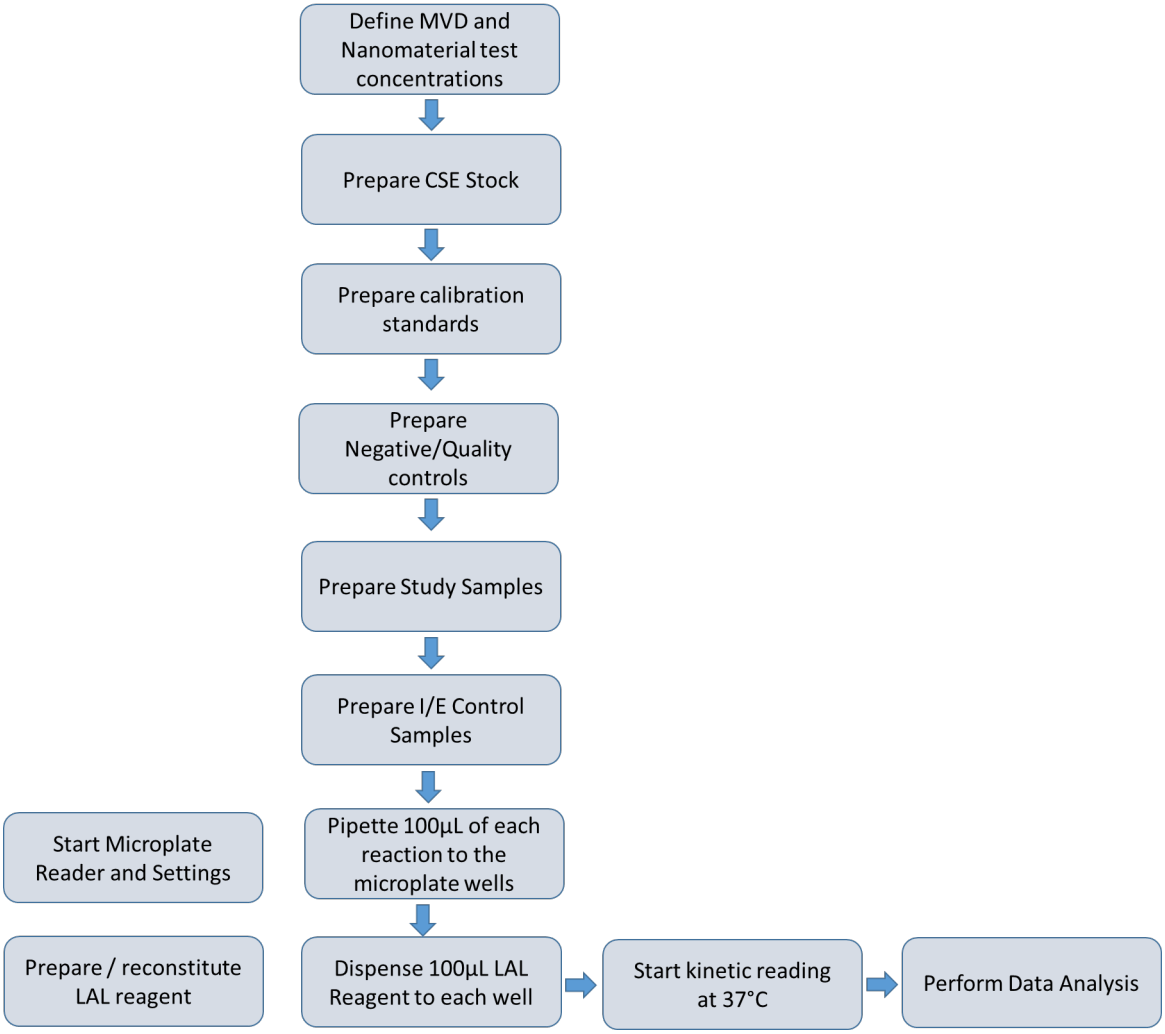

Figure 1: Brief outline of the workflow.

6.4 Measurement Procedure

6.4.1 Test procedure

Turn on the reader instrument approximately 20-30 minutes before starting the assay to allow the instrument to warm up. Set temperature to 37 °C. Switch detection wavelength to 340 nm, and adjust all settings as listed below, or use a preinstalled program which is according to these settings.

Setting for automated temperature controlled Microplate reader:

- 340 nm absorbance
- 37 °C incubation temperature
- 160 – 180 cycles with 45 s cycle time
  (different cycle may be used with total measurement time: approximately 2h)

Alternatively, different cycle times with adapted number of performed cycles may be used with a total reading time of at least 7200 seconds to allow time for samples with low amounts of endotoxin to develop.
Note: some lots of the lysate are less sensitive than others, if the sensitivity of the particular lot is low, the total measurement time may need to be adjusted to 9000s or longer in order to allow the lowest calibrator to develop.

6.4.2 Preparation of the 96-well microplate

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 of an assay designed for 3 test-samples in three different dilutions , each with a PCC, all reactions performed in duplicates. 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	0.5	0.05	0.005	S1.1	S1.1	S1.2	S1.2	S1.3	S1.3	
	5	0.5	0.05	0.005	S1.1 PPC	S1.1 PPC	S1.2 PPC	S1.2 PPC	S1.3 PCC	S1.3 PCC	
	OC	QC	BW	BW	S2.1	S2.1	S2.2	S2.2	S2.3	S2.3	
					S2.1 PPC	S2.1 PPC	S2.2 PPC	S2.2 PPC	S2.3 PCC	S2.3 PCC	
					S3.1	S3.1	S3.2	S3.2	S3.3	S3.3	

					S3.1 PPC	S3.1 PPC	S3.2 PPC	S3.2 PPC	S3.3 PCC	S3.3 PCC	

Shortly before usage reconstitute the necessary LAL Reagent vials with LAL Reagent Water or Glucashield® buffer (according to the manufacturers instruction), mix only gently (do not vortex !) as described above (for details read under -Reagent Preparation-).

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.

Mix gently for about 15 seconds and start automated reading procedure immediately (reading is performed with the microplate cover removed).

During the measurement time, do not disturb the reaction plate. The laboratory bench supporting the optical reader should be free from excessive vibration.

6.5 Data Analysis

The reaction time needed for the appearance of turbidity 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 in EU/mL from the reaction time. The initial absorbance of each well is used as blank for its subsequent kinetic readings to perform a baseline correction and to determine the time to reach an increase of 0.100 absorbance units. The correlation coefficient absolute value for the standard curve should be ≥0.980 to enable a reliable interpolation of unknown samples. The different parameters are the absorption values for the x-range, mean reaction time for the y-range, and 0.100 as threshold value, in order to determine the reaction time for the increase of 0.1 absorbance units. Construct a standard curve by regression of the log onset time against the log endotoxin concentration for the standards. The equation for the regression line describes the standard curve. The line equation of the standard curve is used for calculation of endotoxin concentrations of the samples (including standards and controls). Analysis is performed by the appropriate template of the MARS Data Analysis Software (BMG Labtech). The software is used to directly calculate the results from the microplate reader of the kinetic turbidimetric LAL assay.

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.

7 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 7.1, run should be repeated.
5. If standard curve fails to meet acceptance criterion described in 7.1 – 7.3, 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.

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

CV coefficient of variation

EL Endotoxin Limit

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)

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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.