SCIENCE-BASED RISK GOVERNANCE
OF NANO-TECHNOLOGY

RISK GONE

Consolidated pre-validated guidance document on the determination of ENMs endotoxins content

DELIVERABLE 4.4

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Abstract

The objective of Task 4.1 of RiskGONE project is to develop guidance documents (GDs) for the experimental techniques used to characterize the physicochemical properties of engineered nanomaterials (ENMs). These GDs will be part of a framework for the governance of ENMs and will be later available to the regulatory agencies dealing with this topic.

This deliverable (D4.4) deals with the determination of the endotoxin content of ENMs, building on previous documents, including the European Standard EN ISO 29701, projects experience (NANoREG Deliverable 5.06), and recent literature.

Here we focused on the chromogenic version of the Limulus Amebocyte Lysate (LAL) assay, reviewing the method in relation to the specific characteristics of ENMs. At this aim Round Robin (RR) exercises were organized among RiskGONE partners, with the aim of verifying the validity and the reproducibility of the proposed guidance document. A Standard Operating Procedure (SOP) has been developed, implementing specific conditions and controls for the application of the test to ENMs, based on the test manufacturer's instructions, on the reviewed documents, and according to the experience gained during the RR exercises performed.

The results of the RRs showed that the test is subject to some limitations mainly due to interference of the ENMs with the detection method, and lack of reproducibility of the results among the different laboratories performing the test. In the final section of this document, potential solutions to handle those limitations are discussed.

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List of abbreviations

EU - endotoxin units

FDA - food and drug administration

GDs - guidance documents

EMA - european medicines agency

ENMs - engineered nanomaterials

LAL - Limulus Amebocyte Lysate

LPS - lipopolysaccharides

MAT - monocytes activation test

OD - optical density

OECD - organisation for economic co-operation and development

rFC - Factor C

PMBC - human peripheral blood mononuclear cells

RPT - rabbit pyrogen test

RR - round robin

SOP - standard operation procedure

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1. Introduction

1.1 Background

In the study of the biological and health effects of engineered nanomaterials (ENMs), the particles physicochemical properties are a key aspect that needs to be considered. The immunological safety of ENMs plays an important role especially in some domains, such as nanomedicine and health-related applications. Several ENMs have been reported to trigger inflammatory responses in different *in vitro* and *in vivo* models. Lately, awareness has risen on the possibility of ENMs contamination by bacteria and bacteria components i.e. endotoxins. Endotoxins (or lipopolysaccharides, LPS) are molecules situated on the outer cell membrane of Gram-negative bacteria. They are widespread environmental contaminants, with a high potential of inducing inflammation and toxicity in exposed organisms and biological models (Li et al., 2017). Endotoxins can bind to the surface of ENMs as contaminating agents, leading to misinterpretation of results, or altered results, when it comes to *in vitro* and *in vivo* investigations such as the release of inflammatory cytokines (Li et al., 2017). For this reason, it is of pivotal importance to characterize ENMs for potential endotoxin contamination, in order to properly interpret the results of *in vitro* and *in vivo* investigations.

The objective of Task 4.1 of RiskGONE project is to develop guidance documents (GDs) for the experimental techniques used to characterize the physicochemical properties of ENMs. The GDs will be part of a framework for the governance of ENMs and will be later available to the regulatory agencies dealing with this topic.

This deliverable focuses on the determination of ENMs endotoxin content, building on previous documents including the European Standard EN ISO 29701, projects experience (NANoREG Deliverable 5.06) and relevant literature. At this aim, here we selected the chromogenic version of the Limulus Amebocyte Lysate (LAL assay) as method to be reviewed focusing on the ENMs specific needs, and tested through Round Robin (RR) exercises for its validity and reproducibility.

Several methods for endotoxin determination are available and have been already considered in the past years for their potential suitability in ENMs testing. Both weaknesses and strengths of the various methods have been highlighted in different studies (Hannon and Prina-Mello, 2021; Mangini et al., 2021; Li and Boraschi, 2016; Smulders et al., 2012). The rabbit pyrogen test (RPT) is the oldest method for endotoxin detection, and the first regulatory-approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Being an *in vivo* assay, the use of the RPT rises ethical concerns, besides being costly and time consuming. For these reasons EMA strongly encourages the replacement of this method since 2009 (EMEA/CHMP/BWP/452081/2007), thus this test was not here considered for further validation. The Human Peripheral Blood Mononuclear Cell (PBMC) activation assay and the human Monocytes Activation Test (MAT) measure the inflammatory response of cells to inflammation-inducing agents, without being selective to endotoxins. As a consequence, these assays are not suitable to the use with ENMs with unknown toxicity, as they cannot distinguish between the inflammatory effect induced by contaminants and those that might be intrinsic to the particles (Li et al., 2017). Therefore, also these methods have not been considered here for further validation.

The LAL assay is a fast, sensitive, and endotoxin-specific method that has been largely used. Over the years different readout variants have been developed i.e. i) the gel-clot method, ii) the turbidimetric method, iii) the chromogenic assay (traditional set up with readout at 405 nm, and modified set up with readout at 540 nm), and iv) the fluorescence assay (replacing the Limulus amebocyte lysate with

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the recombinant Factor C (rFC)). The LAL test has been adopted by the standard ISO 29701:2010, while there are no guidance documents provided by the OECD describing this method.
Since the test was originally developed for the use with soluble molecules, the suitability of this method for its use with ENMs is needed, as the ENMs' peculiar physicochemical properties might interfere with the test at different levels (from the enzymatic activity to the readout), therefore leading to false results (Li and Boraschi, 2016).
The ISO 29701:2010 states that the interference potentially generated by the color or by the turbidity of the test sample is a matter of concern and it highlights the importance of pH for the LAL reaction. For a proper validation of the method, the ISO recommends testing a series of dilutions of the test sample, with and without a known amount of spike endotoxin, in order to highlight possible interference.
ENMs interference with the LAL test was explored in previous works and projects (EU project NANoREG D5.06; Li et al., 2015; Smulders et al., 2012; Dobrovolskaia et al., 2014; Dobrovolskaia et al., 2010). All the different readout methods have been reported to have possible interference with ENMs, based on the particles' properties. As a general approach it was recommended to select the test method according to the features of the test samples and the interference observed (Li et al., 2017). However, some evidences suggest that both the gel-clot and the turbidimetric assays might not be optimal for a wide application of endotoxin testing in nano-safety laboratories in a regulatory perspective, due to high interference issues and, respectively, i) low precision and ii) limitations related to the need of specific equipment and competences (NANoREG D5.06; Li et al., 2017).

1.2 RiskGONE approach

Based on the above considerations, the chromogenic version of the LAL assay has been selected for further review and validation within RiskGONE project. Different ENMs have been tested in the RR exercises among the various laboratories involved in the Task, in order to verify the consistency of the results obtained and to develop specific interference controls. NILU and LIST participated to the first RR (RR1) and CSIC joined the work in RR2.

The ENMs tested are summarized in the table below:

ENMs selected in RiskGONE for RRs and the unique identifiers defined in the project

ENM	Identifier
TiO_2 Supplier: Sigma-Aldrich	ERM00000062
ZnO Supplier: Sigma-Aldrich	ERM00000063
TiO_2 Supplier: JRC	ERM00000064
ZnO Supplier: JRC	ERM00000065
MWCNT Supplier: JRC	ERM00000066
Ag nanowires Supplier: Plasmachem	ERM00000067
PLGA-AuNP-WOW (prepared by "WOW" method) Supplier: MyBiotech	ERM00000083
PLGA-AuNP-NP (prepared by NanoPrecipitation method) Supplier: MyBiotech	ERM00000084

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AuNPs-1 (15nm nominal) Supplier: MyBiotech	ERM00000085
AuNPs-2 (50 nm nominal) Supplier: MyBiotech	ERM00000086
CuO Supplier: Plasmachem	ERM00000088
Wo/CO Supplier: NanoAmor	ERM00000089
MWCNT 3 wt% - NC7000 Supplier: Nanocyl	ERM00000325

A Standard Operating Procedure (SOP) was drawn by LIST and NILU, reviewing and adapting the kit manufacturer's instructions and the EN ISO 29701:2010, and trying to better understand and address the interference issue. The first protocol developed for RR1 was tested and further modified to be applied in RR2 by NILU, LIST and CSIC.

The approach applied, technical adaptations and results obtained are reported below, and the final SOP is reported as annex (Annex 1).

2. Performance of RRs and results

2.1 Methods

The Pierce™ Chromogenic Endotoxin Quantitation Kit (Thermo Scientific) was selected among the available kits on the market. The test was performed according to the manufacturer's instructions, and the developed SOP (Annex 1).

The endotoxin content of the samples was calculated as endotoxin units (EU) per volume of reaction (EU/ml), based on the linear regression equation obtained from the standard curve, as shown in the example reported in Fig. 1.

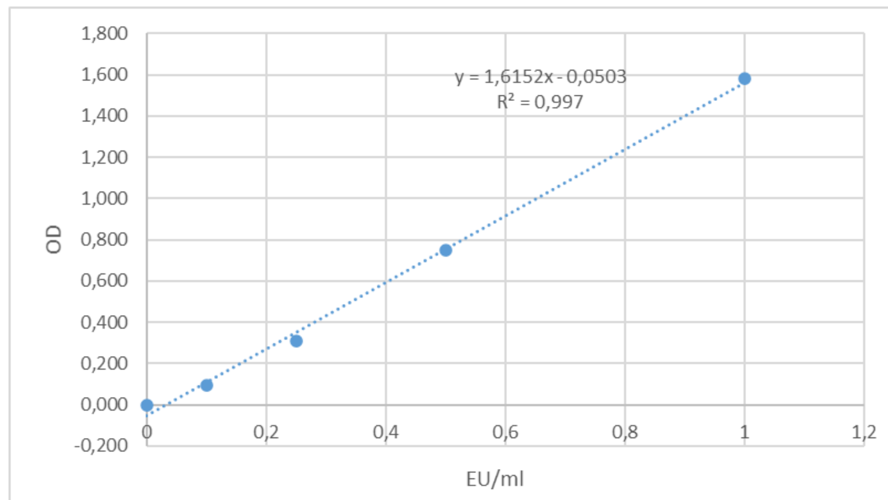

Fig. 1: Representative standard curve and correspondent linear regression equation coupled with R^2 value (>0.98). Optical Density values (OD, y-axis) are plotted against endotoxin concentration values (EU/ml, x-axis).

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The following specific procedures and methods were applied for the ENMs testing:

- Pristine ENMs: at least two (or three when possible) concentrations of each ENM were tested. The concentrations were selected within the range of the exposure conditions used in WP5 for the human hazard assessment *in vitro* studies (1 - 100 µg/ml). This approach enables the validation of the results obtained in nanotoxicology testing.

- Dispersion protocol: the particle suspensions were prepared according to the specific dispersion procedures developed by RiskGONE WP4. For the particles that met the validation requirements (see below), the endotoxin content was then calculated in endotoxin units per mass of ENMs (EU/µg).

- Spiked samples: as test validation control recommended by the kit instructions, the ISO 29701:2010 and previous studies (NANoREG D5.06; Li et al., 2014), the same samples were tested after the addition of a known concentration of endotoxin (spike). The endotoxin recovery rate is thus calculated as follows:

  $$\text{Recovery rate (\%)} = (EU_{\text{sample+spiked endotoxin}} - EU_{\text{sample}}) / EU_{\text{spiked endotoxin}} * 100$$

  where $EU_{\text{sample+spiked endotoxin}}$ is the concentration of endotoxins in the spiked sample as calculated through the regression equation; EU_{sample} is the calculated concentration of endotoxins in the same sample when the spike was not added; $EU_{\text{spiked endotoxin}}$ is the theoretical concentration of endotoxin in the spiked sample.

  A deviation within +/- 25 % of the expected value of endotoxin concentration is considered as a requisite for the test validation, according to the manufacturer's instructions. However, according to the ISO 29701:2010, the recovery rate should be within 50 to 200 % of the nominal value. Since the suggested values are not in agreement, both thresholds have been included in the template for data collection, allowing a more detailed analysis of the results, but the ISO acceptance criteria were here applied and considered as reference for the validation.

- Mock samples: the absorbance of the ENMs suspensions was measured at the wavelength used for the test reading (405 nm), to take into account the possible interference with the readout of the test. This additional verification step is not described by the ISO 29701:2010, but it is recommended by the LAL kit manufacturer, in order to determine if the sample's intrinsic colour could alter the absorbance measurement.
  The mock samples were prepared by adding endotoxin free water to the ENM suspensions, instead of the kit reagents. The stop solution was also finally added to the mixture. The manufacturer's instructions state that absorbance values significantly different from the blank (made by endotoxin-free water and the stop reagent), indicate that the intrinsic colour of the test substance can alter the correct sample absorbance measurements. No further indications are given about the extent of that difference to be significant. Thus, we established our internal threshold, considering the absorbance being significantly different if the deviation from the blank was +/- 25 %.
  The absorbance spectrum of the mock samples was also read between 300 and 600 nm.

- Depyrogenated samples: depyrogenation by heat treatment was tested as a way to remove a potential endotoxin contamination from the ENMs. Briefly, the ENMs were dry heat treated at

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180 °C for 3 h, or 250 °C for 30 min before testing. Then ENM suspensions were prepared following the dispersion protocol developed within RiskGONE WP4 and investigated through LAL chromogenic assay. The results derived from depyrogenated samples were compared with those obtained by the correspondent non-depyrogenated ENM. The detection of a lower endotoxin content in depyrogenated samples compared to the non-depyrogenated ones might indicate a contamination by endotoxins in the sample, and would help to exclude false positive due to ENM colour interference.

2.2 Results

RR1

The partners participating to RR1 were LIST and NILU. In RR1 the following ENMs were tested: ERM00000062 (TiO_2 Sigma-Aldrich), ERM00000063 (ZnO Sigma-Aldrich), ERM00000064 (TiO_2 JRC), ERM00000065 (ZnO JRC), ERM00000067 (AgNWs), ERM00000083 (PLGA-AuNP-WOW), ERM00000085 (AuNPs-1), ERM00000086 (AuNPs-2). Two ENMs concentrations were used: 10 and 100 µg/ml.

The High standard curve (as defined by the manufacturer's instruction) was selected to run the first trial (0.1-1 EU/ml). The samples and the validation controls were prepared and run as described in the method section (2.1) and in the SOP (Annex 1). The results obtained from each experiment were collected in a template specifically developed by the partners. A representative example of this template is reported in Fig. 2.

In particular, the template contains the following parameters, calculations and validation queries:

- the OD of the mock samples are reported ("OD mock" column in Fig. 2) and compared to the OD of the blank ("readout interference" column). The readout interference is considered to be significant if the ENMs suspension absorbance differed from the blank by +/- 25 %.
- EU/ml are calculated based on the linear regression equation obtained by the standard curve. R^2 must be >0.98.
- The recovery rate is calculated comparing the spiked samples to the normal ENMs samples as described above (section 2.1 Methods)
- The acceptance criteria of the kit (manufacturer instruction) and those of ISO are reported.
- For the samples satisfying the i) readout interference control (negative = N in Fig. 2) and ii) "validation controls" based on ISO requirements (acceptance = yes, Y in Fig. 2), the endotoxin content per ENM mass (EU/µg) is calculated.
- The last column, "Endotoxin contamination", indicates the presence of measurable levels of endotoxin, considering the sensitivity of the assay, i.e.
  - if the test is valid, and the EU measured falls within the range of the standard curve, the template reports "Y" (yes, endotoxin contamination present)
  - if the test is valid, but the EU measured falls below the range of the standard curve, the template reports "N" (no, endotoxin contamination not present)

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- if the test is valid, but the EU measured falls above the range of the standard curve, the template reports "Y*" (yes, endotoxin contamination present → need to repeat the test using a standard curve in a higher concentrations range, for more precise results)
- if the test is invalid, the template reports "NA" (not available, no answer)

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				OD mock	Readout interference (+/- 20%)	OD LAL		Blank subtraction		EU/ml		EU/ml average	SD	Recovery rate (%)	Acceptance (kit= +/- 25 %)	Acceptance (ISO= 50<>200%)	EU/ug	Endotox contamin. Y/N
						R1	R2	R1	R2	R1	R2							
			st1=1			1,934	1,783	1,658	1,506	1,047	0,956	1,002	0,064					
			st2=0.5			1,114	0,933	0,838	0,656	0,555	0,446	0,500	0,077					
			st3=0.25			0,616	0,558	0,340	0,282	0,256	0,221	0,238	0,025					
			st4=0.1			0,391	0,355	0,114	0,079	0,120	0,099	0,110	0,015					
			Blank	0,259	0,1940625<x<0,3234375	0,267	0,285	-0,009	0,009	0,046	0,057	0,052	0,008					
			Blank average			0,276315												
	ug/ml	Depyrogen.	Spiked endotox															
ERM00000085 (AuNPs-1)	100	N	N	0,315	N	3,513	3,559	3,237	3,282	1,996	2,023	2,009	0,019	4,114	N	N	invalid	NA
	100	N	Y	0,315	N	3,544	3,596	3,268	3,320	2,014	2,045	2,030	0,022					
	100	Y	N	0,258	N*	2,402	2,430	2,126	2,154	1,328	1,345	1,337	0,012	-3,015	N	N	invalid	NA
	100	Y	Y	0,258	N*	2,407	2,375	2,130	2,099	1,331	1,312	1,322	0,013					
ERM00000086 (AuNPs-2)	100	N	N	0,392	Y	3,535	3,315	3,259	3,039	2,009	1,877	1,943	0,093	6,703	N	N	invalid	NA
	100	N	Y	0,392	Y	3,415	3,546	3,139	3,270	1,937	2,015	1,976	0,056					
	100	Y	N	0,278	N*	0,330	0,349	0,053	0,073	0,084	0,095	0,089	0,008	100,043	Y	Y	invalid	NA
	100	Y	Y	0,278	N*	1,157	1,187	0,881	0,911	0,581	0,599	0,590	0,013					
ERM00000063 (ZnO Sigma-Aldrich)	100	N	N	0,250	N	0,277	0,293	0,001	0,016	0,052	0,061	0,057	0,007	5,824	N	N	invalid	NA
	100	N	Y	0,250	N	0,327	0,340	0,051	0,063	0,082	0,090	0,086	0,005					
	100	Y	N	0,288	N	0,280	0,277	0,004	0,000	0,054	0,052	0,053	0,001	147,836	N	Y	0,000527327	N
	100	Y	Y	0,288	N	1,448	1,570	1,172	1,293	0,756	0,828	0,792	0,051					
ERM00000062 (TiO2 Sigma-Aldrich)	100	N	N	0,277	N	0,285	0,273	0,009	-0,003	0,057	0,050	0,053	0,005	95,915	Y	Y	0,000532102	N
	100	N	Y	0,277	N	1,026	1,129	0,749	0,853	0,502	0,564	0,533	0,044					
	100	Y	N	0,297	N	0,274	0,289	-0,002	0,013	0,050	0,059	0,055	0,006	82,305	Y	Y	0,000547898	N
	100	Y	Y	0,297	N	0,971	0,963	0,695	0,686	0,469	0,464	0,466	0,004					
ERM00000064 (TiO2 JRC)	100	N	N	0,401	Y	0,418	0,431	0,141	0,154	0,136	0,144	0,140	0,006	77,963	Y	Y	invalid	NA
	100	N	Y	0,401	Y	1,148	0,999	0,871	0,723	0,575	0,486	0,530	0,063					
	100	Y	N	0,306	N*	0,305	0,308	0,028	0,032	0,069	0,071	0,070	0,001	93,017	Y	Y	invalid	NA
	100	Y	Y	0,306	N*	1,068	1,093	0,792	0,817	0,527	0,542	0,535	0,010					

Fig. 2: Representative data collection template showing NILU results from RR1, exp 1. * depyrogenated samples with unknown particles concentration, see the text “Interpretation of results”

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In addition, the absorbance spectrum of the mock samples was read between 300 and 600 nm, to better investigate possible interference with the readout of the test. Below (Fig 3.), few representative spectra are shown:

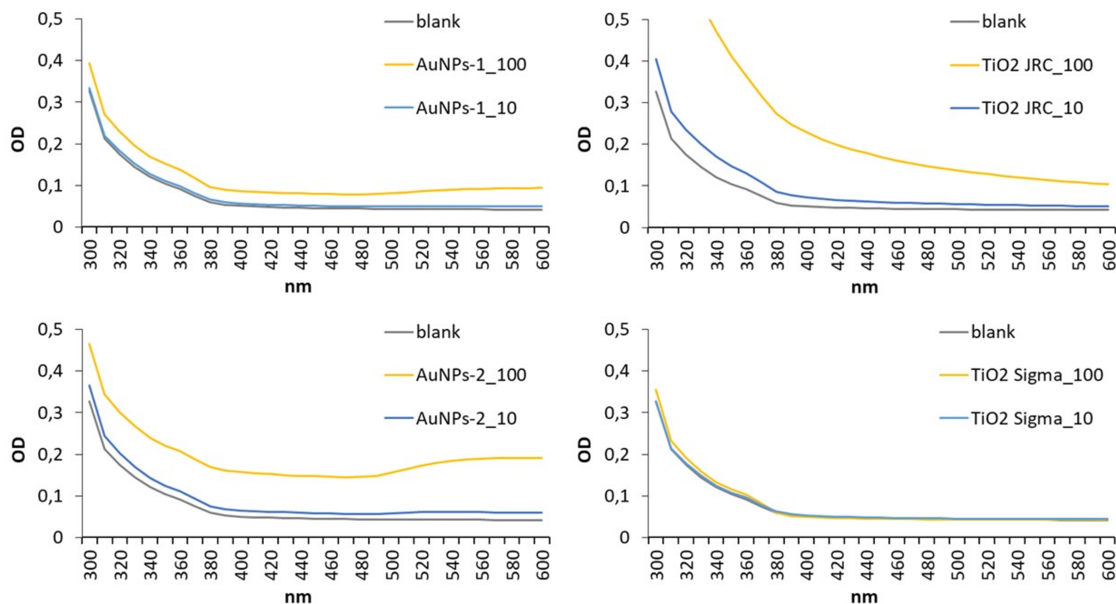

Fig. 3: absorbance spectra of ENMs suspensions at concentrations 10 and 100 μg/ml.

In RR1, two independent experiments were run at NILU and one at LIST. A summary of the results obtained by the partners in RR1 is reported in Fig. 4.

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NP identifier	ug/ml	Depyrogenation	Readout interference (+/- 25%)			Kit acceptance criteria (+/- 25%)			ISO acceptance criteria (50%<x<200%)			EUendotoxins/ugENM (invalid IF acceptance criteria not met)			Endotoxin contamination (Y/N/Y*)		
			LIST_RR1	NILU RR1_2	NILU RR1_1	LIST_RR1	NILU RR1_2	NILU RR1_1	LIST_RR1	NILU RR1_2	NILU RR1_1	LIST_RR1	NILU RR1_2	NILU RR1_1	LIST_RR1	NILU RR1_2	NILU RR1_1
ERM00000085 (AuNPs-1)	100	N			N			N			N			invalid			NA
	100	Y			N*			N*			N*			invalid			NA
	10	N		N		N	N		N	N		invalid	invalid		NA	NA	
ERM00000086 (AuNPs-2)	100	N			Y			N			N			invalid			NA
	100	Y			N*			Y*			Y*			0,00089384			N
	10	N		N		N	N		N	Y		invalid	0,26920236		NA	Y*	
ERM00000083 (PLGA-AuNP-WOW)	10	N		N			N			N			invalid			NA	
ERM00000063 (ZnO Sigma-Aldrich)	100	N		N	N		N	N		N	N		invalid	invalid		NA	NA
	100	Y			N			N			Y			0,00052733			N
	10	N		N			N			N			invalid			NA	
ERM00000065 (ZnO JRC)	100	N		N		N	N		N	Y		invalid	0,00054378		NA	N	
	10	N		N			Y			Y			0,00632182			N	
ERM00000062 (TiO2 Sigma-Aldrich)	100	N		N	N	N	Y	Y	Y	Y	Y	invalid	0,00143127	0,0005321	NA	Y	N
	100	Y			N			Y			Y			0,0005479			N
	10	N		N			Y			Y			0,0084059			N	
ERM00000064 (TiO2 JRC)	100	N		Y	Y	N	Y	Y	N	Y	Y	invalid	invalid	invalid	NA	NA	NA
	100	Y			N*			Y			Y			0,00069622			N
	10	N		N			Y			Y			0,01004572			Y	
ERM00000067 (AgNWs)	100	N		N			N			Y			0,00198738			Y	
	10	N		N			Y			Y			0,01153665			Y	

Fig. 4: Summary of results from the RR1 endotoxin test from NILU and LIST, and consistency analysis (green = consistency, red = inconsistency). *depyrogenated samples with unknown particles concentration, see the text “Interpretation of results”.

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Interpretation of results and considerations from RR1

Many particles showed interference either with the readout (mock samples control) or at other levels (spiked samples validation control) when tested at the highest concentration. Thus, the test resulted to be invalid for ENMs e.g. TiO_2 (JRC), AuNPs-1, AuNPs-2 (100 µg/ml) and PLGA-AuNP-WOW.

Interference readout can be easily highlighted by running the mock samples controls at the wavelength used for the detection (405 nm). Monitoring the ENMs suspensions absorbance spectrum can also give an indication about a potential interference with the modified version of the chromogenic LAL test with readout at 540 nm. For example, the spectrum reported in Fig. 3 for the higher concentration (100 µg/ml) of AuNPs-2 and TiO_2 (JRC) suggest that also the modified version of the chromogenic LAL test would not be suitable for these particles.

The dilution of the sample is indicated as a way to overcome the interference issue, and indeed some particles, e.g. TiO_2 (JRC) and AuNPs-2, presented interference when tested at 100 µg/ml, but not when they were diluted to 10 µg/ml (Fig. 4). This approach didn't work with all the ENMs, as ZnO (Sigma-Aldrich) and AuNPs-1 still interfered with the test at the lower concentration tested.

Interestingly, some samples (e.g. AuNPs-1 at 10 µg/ml) didn`t show a significantly different absorbance from the blank at the test readout wavelength, but failed the endotoxin recovery validation control (Fig. 4), suggesting that this interference might be due to other phenomena probably affecting the LAL reaction.

It is worth noticing that the samples dilution as strategy to avoid ENMs interference might not always be a valid method, as it might result in a final endotoxin concentration lower than the test detection limit (test sensitivity). In this case a possible endotoxin contamination would not be detected. If the toxicological investigations have to be performed using higher ENMs concentrations compared to those established for the LAL testing, uncertainty will remain concerning the role of potentially present endotoxins on the toxic effects observed.

In RR1 some test samples fulfilled the validation control step only when the criteria indicated by the ISO 29701:2010 were applied (endotoxin recovery rate 50 - 200 % of the expected value), although they failed the more stringent criteria indicated by the kit manufacturer's instructions (+/- 25%). For the specific purposes of RiskGONE WP4 RR exercises, we decided to apply the criteria indicated by the ISO, since it is the only official guidance available at the moment. However, some discussion might be needed in order to revise and select the best validation criteria.

Depyrogenation by dry heat treatment was applied in RR1 exercise as described above, as a way to remove any potential endotoxin contamination present on the ENMs. Each ENM was tested in both the forms, as depyrogenated or non-depyrogenated samples. This approach revealed major drawbacks especially when ENMs already provided as suspensions were used. During the depyrogenation process the suspensions dry out, and the particles attached to the vial's walls are not easily detached to be re-suspended in endotoxin free water. Thus, the concentration of the depyrogenated samples is underestimated in some cases (* in Fig. 2 and 4). This might explain the fact that some depyrogenated samples fulfilled the readout validation requirements while the original samples didn't. However, the results obtained from these samples are not reliable since their particles concentration is unknown. The depyrogenation technique might present issues even for particles originally provided as powder. The heat treatment at high temperatures might induce changes on the particles properties (effect particle-specific) and these modifications need to be evaluated on a case-by-case basis. For the purpose of this deliverable, we thus considered the depyrogenation approach as not suitable for the

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selected ENMs. In support to our considerations, Li and colleagues (2017) also concluded on the unsuitability of this treatment when applied to ENMs, due to potential changes in the particles physicochemical properties.

The protocol used in RR1 was reviewed and modified based on the experience obtained during the first exercise to be then applied in the RR2. For the reasons explained above, the depyrogenation approach was not used in RR2, while the established acceptance criteria (mock samples OD, validation control steps) were considered to be robust enough to provide valuable results and were thus kept in the final SOP.

Additional particles were selected to be tested within RR2 in agreement with WP5 activities.

RR2

The partners participating to RR2 were NILU, LIST and CSIC. In RR2 the following ENMs were tested: ERM00000062 (TiO2 Sigma-Aldrich), ERM00000063 (ZnO Sigma-Aldrich), ERM00000064 (TiO2 JRC), ERM00000065 (ZnO JRC), ERM00000067 (AgNWs), ERM00000083 (PLGA-AuNP-WOW), ERM00000085 (AuNPs-1), ERM00000086 (AuNPs-2), ERM00000088 (PL-CuO), ERM00000089 (Wo/CO), ERM00000325 (MWCNT 3wt% - Nanocyl). At least two (or three when possible) ENMs concentrations were tested: (1), 10 and 100 µg/ml.

In RR1 some samples were labelled as "N" (endotoxin contamination not present) under the "Endotoxin contamination" query, highlighting that the test was valid, but the EU measured fell below the range of the High standard curve. Therefore, in RR2 the lower standard curve ranging within 0.01-0.1 EU/ml was applied to increase the sensitivity of the test.

The samples and the validation controls were prepared and analysed as previously described, and following the final SOP (Annex 1). The results from each experiment were collected in the template developed from the partners after RR1, and described in the previous section. No further updates to the template were considered as necessary.

In RR2, each partner (NILU, LIST and CSIC) run one independent experiment. The summary of the results obtained by the partners are reported in Fig. 5. Fig. 6 shows an intra- and interlaboratory comparison of the results (endotoxin contamination query) from RR1 and RR2 from NILU, LIST, and CSIC.

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NP name/code	ug/ml	Depyrogen.	Readout interference (+/-25%)			Acceptance (kit= +/- 25 %)			Acceptance (ISO= 50<>200%)			EU/ug			Endotox contamin. Y/N (based on detection limit of the test)		
			CSIC_RR2	LIST_RR2	NILU RR2	CSIC_RR2	LIST_RR2	NILU RR2	CSIC_RR2	LIST_RR2	NILU RR2	CSIC_RR2	LIST_RR2	NILU RR2	CSIC_RR2	LIST_RR2	NILU RR2
ERM00000085 (AuNPs-1)	100	N															
	10	N	N			N			N			invalid			NA		
ERM00000086 (AuNPs-2)	100	N															
	10	N	N			N			Y			0,0002816			N		
ERM00000083 (PLGA-AuNP-WOW)	10	N	Y			N			N			invalid			NA		
ERM00000063 (ZnO Sigma-Aldrich)	100	N	N	N	N	N	N	N	Y	N	N	-0,0006482	invalid	invalid	N	NA	NA
	10	N	N	N	N	N		N	Y		N	-0,0047091		invalid	N		NA
ERM00000065 (ZnO JRC)	100	N		N													
	10	N		N			Y			Y			-0,002467			N	
ERM00000062 (TiO2 Sigma-Aldrich)	100	N	Y	N		N	N		Y	Y		invalid	0,000342		NA	Y	
	10	N	Y	N		Y	N		Y	Y		invalid	0,0012126		NA	Y	
ERM00000064 (TiO2 JRC)	100	N	Y	Y	Y	Y	N	N	Y	Y	Y	invalid	invalid	invalid	NA	NA	NA
	10	N	Y	N	Y	Y	N	Y	Y	Y	Y	invalid	0,0012376	invalid	NA	Y	NA
ERM00000067 (AgNWs)	100	N	Y		Y	N		N	N		N	invalid		invalid	NA		NA
	10	N	N		N	N		Y	Y		Y	-0,0003772		0,0018535	N		Y
ERM00000089 (Wo/CO)	100	N	Y	N	N	N	Y	N	N	Y	Y	invalid	0,0009408	0,0015864	NA	Y	Y*
	10	N	N	N	N	N	N	Y	N	Y	Y	invalid	0,0007942	0,0011006	NA	N	N
ERM00000088 (PL-CuO)	100	N	Y	N	N	N	Y	N	Y	Y	Y	invalid	6,88E-05	0,0001332	NA	N	Y
	10	N	Y	N	N	N	Y	N	Y	Y	Y	invalid	0,0004225	0,001103	NA	N	N
RM00000325 (MWCNT 3wt% - Nanocy	100	N	Y	Y	Y	Y	N	Y	Y	N	Y	invalid	invalid	invalid	NA	NA	NA
	10	N	Y	Y	Y	N	N	N	N	N	Y	invalid	invalid	invalid	NA	NA	NA

Fig. 5: Summary of results from the RR2 endotoxin test from NILU, LIST, and CSIC, and consistency analysis (green = consistency, red = inconsistency).

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NP identifier	ug/ml	Endotoxin contamination (Y/N/Y*)			Endotoxin contamination (Y/N/Y*)			
		LIST_RR1	NILU RR1_2	NILU RR1_1	CSIC_RR2	LIST_RR2	NILU RR2	
ERM00000085 (AuNPs-1)	100			NA				
	100			NA				
	10	NA	NA		NA			Interlaboratory Consistency between RR1 and RR2
ERM00000086 (AuNPs-2)	100			NA				
	100			N				
	10	NA	Y*		N			
ERM00000083 (PLGA-AuNP-WOW)	10		NA		NA			Interlaboratory Consistency between RR1 and RR2
ERM00000063 (ZnO Sigma-Aldrich)	100		NA	NA	N	NA	NA	Interlab&Intralab Consistency between RR1 and RR2
	100			N				
	10		NA		N		NA	Intralaboratory Consistency between RR1 and RR2
ERM00000065 (ZnO JRC)	100	NA	N					
	10		N			N		Interlaboratory Consistency between RR1 and RR2
ERM00000062 (TiO2 Sigma-Aldrich)	100	NA	Y	N	NA	Y		
	100			N				
	10		N		NA	Y		
ERM00000064 (TiO2 JRC)	100	NA	NA	NA	NA	NA	NA	Interlab&Intralab Consistency between RR1 and RR2
	100			N				
	10		Y		NA	Y	NA	

Fig. 6: Intra- and interlaboratory comparison of results (endotoxin contamination query) from RR1 and RR2 from NILU, LIST, and CSIC

Interpretation of results and considerations from RR2

As for RR1, the results reported from the partners were in agreement for most of the samples, even if some inconsistencies were also found.

Agreement was also observed in some cases with the results reported in RR1. For example AuNPs-1 at 10 µg/ml didn`t show any readout interference, but failed the endotoxin recovery validation control (data by CSIC in RR2, Fig. 5; data by NILU and LIST in RR1, Fig. 4).

In general, all the partners agreed in reporting interference issues with the gold particles (AuNPs-1, AuNPs-2 and PLGA-AuNP-WOW), TiO_2 (JRC) and the MWCNT. It was also again observed that diluting the sample might overcome interference issues (e.g. see results for AgNWs in Fig. 5), but questions remain about possible underestimation of the endotoxin content in relation to the test sensitivity.

No full agreement could be reached on the other ENMs with regard to the fulfilment of the test acceptance criteria and the endotoxin contamination query.

Considering the final parameter "endotoxin contamination" here evaluated, the results obtained from RR1 and RR2 were quite in agreement in an intra- and interlaboratory comparison (see Fig. 6).

2.3. Other considerations

As additional strategy to decrease the risk of interference potentially generated by the presence of particle in the suspension during the readout, the possibility of filtering the ENM suspensions prior testing or just before the measurement was also evaluated. This additional step would allow to retrieve the endotoxins potentially present in the test sample into a ENMs-free solution. The suggested procedure consists of ultrafiltration of the ENMs dispersion by a 30 min centrifugation at 15000g using ultrafiltration vials e.g. Millipore Amicon Ultra-2 Centrifugal Filters (3KDa MWCO), However, some drawbacks in this approach have been identified. Endotoxins have been reported to form a firm and

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stable binding to ENMs, surfaces and materials (Mangini et al., 2021; Li et al., 2017). Thus, at least part of the endotoxin content might not detach from the ENMs during the incubation and centrifugation steps, while another part might bind to the filter and escaping the retrieving in the filtered solution. It was also reported that the capacity of binding endotoxins is ENM-specific and depends on some nanomaterial physicochemical features, including 1) the ability to form Coulomb and van der Waals interactions; 2) the particles aggregation state; 3) the presence of surface functionalization; 4) the pH of the suspension and 5) the ionic strength (Li and Boraschi, 2016).

Although the approach looked promising for some aspects it needs further and deep investigations since it might bring to some misinterpretations of the results due to the i.e. i) unknown amount of endotoxins present in the ENMs, ii) unknown retrieving efficiency by ultrafiltration, iii) unknown amount of endotoxins lost in the filtration plastic ware. Therefore, for the purpose of this specific deliverable (D4.4), the method was not applied.

It has been reported that the LAL assay can experience interference from beta-glucans (NANoREG D5.06). The recombinant Factor C (rFC) test was developed for these cases, as it only measures the interaction between endotoxin and Factor C in isolation, overcoming false-positive results related to other blood components (Factor G) (Ding & Ho, 2001; Grallert et al., 2011). Commercially available kits are based on measuring the fluorescence resulting from the endotoxin-activated rFC interacting with a fluorogenic substrate (excitation at 380 nm and emission at 440 nm). This approach might thus be considered in case interference by the presence of beta-glucans is suspected. Still this test is based on the readout of a fluorescence signal, which is known to be subject to readout interference by ENMs.

3. Conclusions

Different acceptable endotoxin levels are defined by the regulatory authorities depending on the domain and the application of the “endotoxin-containing” products. Regulatory authorities define the maximum tolerated level of endotoxins in drugs or surgical instruments for human use as 0.5 EU/kg, while for products coming in contact with the cerebrospinal fluid, the limit is set at 0.02 EU/kg (Chapter 85, Unites States Pharmacopoeia 2007). Increased levels of mRNA expression for IL-8 were observed after exposure to a very low dose of LPS (0.001 ng/mL) and levels of IL-1 β and TNF-α were detectable after stimulation with doses of 0.01 ng/mL (Zhong et al., 1993). Since ENMs are often applied in the nanomedicine and health related domains, it is crucial to set up a reliable method for endotoxin quantification in ENMs, free from potential interferences due to the interactions between the particles and the different components or techniques foreseen by the commercial kits.

So far, a suitable method that could be widespread applied for endotoxin detection and quantification has not been developed, as all the existing techniques present different drawbacks depending on the properties of the ENMs investigated. Interference of the particles with the test methods have been largely reported and here observed during the RR exercises. Thus, the choice of the most convenient detection method has to be done on a case-by-case basis, possibly considering the already available data on the different ENMs, when available, and proper validation controls for specific cases.

This deliverable provides a protocol for the application of the chromogenic LAL assay to the testing of ENMs, describing the necessary validation controls to deal with interference issues at different levels.

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As described by the kit manufacturer's instruction, the protocol looks very detailed and easy to apply. However, as previously stressed, its application for the endotoxin detection and quantification in ENMs requires additional control samples to validate the obtained results. The agglomeration state of the particles and their size might change if incorrect handling and sonication procedures are followed thus affecting either the readout or the ability to trap endotoxins on their surface. In this perspective a preparatory training of personnel might be beneficial to work on this test with ENMs and the specific experimental conditions here described, especially about the sample's preparation. This might ensure the correct performance of the test and help reducing both inter- and interlaboratory variability.

A data collection template has been here developed, including a decision table for the interpretation of the results, which takes into consideration how the nature of the ENMs could affect the readout (mock control) and how the acceptance criteria might condition the final output (kit vs ISO acceptance limits). The approach here developed, and the SOP reported, allow for the detection of possible interference and for the validation of the results.

4. Deviations from Description of Action

No major deviation to be reported.

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Annex 1

DETERMINATION OF ENDOTOXIN CONTAMINATION OF ENMs BY LAL ASSAY

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0.2	10.11.2020	Elisa Moschini (LIST), Eleonora longhin (NILU)	Draft updated based on RR1	
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Abstract

This is a Standard Operating Procedure (SOP) developed within the RiskGONE project for the *Limulous Amebocyte Lysate* assay (LAL assay) applied to engineered nanomaterials (ENMs). The SOP is based on the Pierce^{TM} Chromogenic Detection kit A3952 (ThermoFisher) and it is developed on the basis of the manufacturer instruction. Specific conditions and controls for the particular use of the test with ENMs have been developed cosidering the NBN EN ISO 29701 (2010), previous experience from the EU project NANoREG (Deliverable 5.06), scientific papers and the experience gained during the different Round Robin (RR) exercises performed within RiskGONE.

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1. BACKGROUND AND PRINCIPLE OF THE TEST

The presence of endotoxin or lipopolysaccharides (LPS) components of Gram-negative bacterial cell walls on inhaled nanoparticles could cause immuno-toxicity such as fevers and disease in case of *in vivo* exposure or could affect the biological responses highlighted in *in vitro* systems, leading to a misinterpretation of the final results. Therefore, the preliminary detection of endotoxins is strongly recommended to exclude the presence of endotoxins in the test sample and for a most adequate interpretation of data obtained by using *in vitro* biological test systems.

The Limulous amebocyte Lysate assay (LAL assay) is commonly used to qualitatively or quantitatively detect endotoxins in samples and it is based on the use of an aqueous extract of amebocyte blood cells from Atlantic horseshoe crab, which reacts with LPS. The test is suitable for the use with nanomaterial samples dispersed in aqueous media, e.g. water, serum or reaction medium, and to such media incubated with nanomaterials for an appropriate duration at 37 °C (NBN EN ISO 29701 (2010).

Bacterial endotoxin catalyses the activation of a proenzyme in the modified Limulus Amebocyte Lysate. The activated proenzyme then catalyses the splitting of p-Nitroaniline (pNA) from the colourless substrate, Ac-Ile-Glu-Ala-Arg-pNA; the activation rate is proportional to the sample endotoxin concentration. After stopping the reaction, the released pNA is photometrically measured at 405-410nm. The correlation between the absorbance and the endotoxin concentration is linear in the 0.01-0.1 or 0.1-1.0EU/mL ranges (Pierce™Chromogenic Detection kit - A3952; Thermofisher), depending on the required sensitivity. The developed colour intensity is proportional to the amount of endotoxin present in the sample and can be calculated using a standard curve obtained using different endotoxin concentrations from E.coli (Endotoxin Standard stock).

2. SAFETY RULES

- Operators must wear lab coat, goggles, and gloves
- Discard exhausted reagents in the appropriated biological wastes collection bin
- Work under a laminar flow hood with HEPA filters

3. GENERAL NOTES/GOOD PRACTICES

- Clean the floor of the laminar flow hood as well as all the tools which go under the flow with ethanol 70%
- Equilibrate all reagents to room temperature before use.
- Equipment and laboratory glassware used for the preparation of the test samples (e.g., pipette tips, glass tubes, microcentrifuge tubes and disposable 96-well microplates) must be endotoxin-free. When commercial products are not available, items should be treated by heating at a temperature higher than 250 °C for at least 30 min or using other validated combinations temperature/time (e.g. 180 °C for at least 3 h, or 650 °C for 1 min) to eliminate endotoxins.
  Heat-labile products or other materials which are not suitable for heat-treatment shall be treated with different methods (e.g. rinsing with endotoxin-free water after soaking the materials in strong alkali or oxidizing solution. In this case, you need to prove that the

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procedure is effective against endotoxins and that no residuals remained after treatment that could interfere with the test (NBN EN ISO 29701 (2010)).

- Use a repetitive pipettor to ensure normalized volumes between samples.
- To stop all bacteriological activity, i.e. if applicable store test samples at 2-8°C for <24 hours or -20°C for >24 hours.
- Maintaining the correct temperature is critical for reproducibility. Use a proper heating block at 37°C±1°C.
- Do not use a cabinet-style incubator to perform the assay.
- Vortex all the solutions before pipetting and use to ensure the correct endotoxin concentrations are measured. Endotoxin adheres to glass and plastic surfaces.
- Glass tubes are preferred for making standard stock solutions; however, polystyrene or polypropylene microcentrifuge tubes (1.5mL) may also be used.
- When using microcentrifuge tubes, dedicate the bag of tubes for the assay and follow aseptic techniques.
- Components of undiluted serum interfere in the assay. Serum samples must be diluted 50- to 100-fold to be compatible. The serum must be completely free of RBCs, and the diluted sample may need to be heat-shocked (70°C for 15 minutes).
- 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.
- Some LAL-reactive glucans (LRGs) may result in false positive signal in the LAL assay. In samples where LAL-reactive glucans may be present, researchers may want to compare cellulase-treated samples to untreated samples to determine contribution of signal resulting from LRGs.
- Samples turning yellow after addition of the Stop Reagent (25% acetic acid) or possessing significant initial colour may require special attention. See the interference section for more details.

4. EXPERIMENTAL PART

4.1. EQUIPMENT NOT PROVIDED

- Heating block at 37°C±1°C
- Laminar flow hood (with HEPA filters)
- Fridge and freezer
- Centrifuge
- Microplate reader

4.2. MATERIAL NOT PROVIDED

- Sterile and pyrogen free aspiration pipettes
- Sterile and pyrogen free plastic pipettes: 5, 10, 25 and 50 ml
- Low retention, sterile and pyrogen free tips: 0.5-10 µl, 20-200 µl, 30-300 µl and 100-1000 µl
- Micropipettes
- Multichannel pipette

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- Pipette controller
- Repetitive pipettor
- Sprayer for ethanol
- Pyrogen-free reservoir
- Sterile and pyrogen free microcentrifuge tubes 1,5 ml
- Pierce™ Chromogenic Endotoxin Quant Kit (Thermo-Fischer, A39552)
- Disposable endotoxin-free 96-well microplates or plate strips

4.3. REAGENTS NOT PROVIDED

- Ethanol 70% (prepared by dilution from absolute ethanol)
- 25% Acetic Acid (stop solution)
- Nanoparticles, engineered nanomaterials (ENMs)
    - For RR1: ERM00000062 (TiO2 Sigma-Aldrich), ERM00000063 (ZnO Sigma-Aldrich), ERM00000064 (TiO2 JRC), ERM00000065 (ZnO JRC), ERM00000067 (AgNWs), ERM00000083 (PLGA-AuNP-WOW), ERM00000085 (AuNPs-1), ERM00000086 (AuNPs-2)
    - For RR2: ERM00000062 (TiO2 Sigma-Aldrich), ERM00000063 (ZnO Sigma-Aldrich), ERM00000064 (TiO2 JRC), ERM00000065 (ZnO JRC), ERM00000067 (AgNWs), ERM00000083 (PLGA-AuNP-WOW), ERM00000085 (AuNPs-1), ERM00000086 (AuNPs-2); ERM00000088 (PL-CuO); ERM00000089 (Wo/CO); ERM00000325 (MWCNT Nanocyl)

4.4. MATERIAL PREPARATION

Endotoxin Standard Stock Solutions

- Each E. coli Endotoxin Standard vial contains 10-50 EU of lyophilized endotoxin; the actual potency is printed on the label. Reconstitute with room temperature Endotoxin-Free Water by adding 1/10 mL of the EU amount indicated on the vial to make Endotoxin Standard (ES) Solution at 10 EU/mL (e.g., a vial with potency of 15 EU, when reconstituted with 1.5 mL of Endotoxin-Free Water (EFW), will yield a concentration of 10 EU/mL).

- Vortex the solution vigorously for 15 minutes (recommended <1500 rpm)

Note: Reconstituted stock solution is stable for 4 weeks at 2-8°C. Prior to subsequent use, warm the solution to room temperature and vigorously mix for 15 minutes. This is important because the endotoxin adheres to the sides of the glass vial.

- Prepare High Standards (0.1-1.0 EU/mL) (Table 1) or Low Standards (0.01-0.1 EU/mL) (Table 2) from the Endotoxin Standard Solution (10 EU/mL) using the dilutions and procedures in Tables 1 and 2. Select the High or Low Standard curve based on the expected content of endotoxin in your samples and the needed sensitivity.

Table 1. Dilutions and procedures for preparing High Standard Stock Solutions (0.1-1.0 EU/mL)

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Vial	Volume of Endotoxin Standard Solution (ml)	Volume of Standard 1 (ml)	Endotoxin-Free Water (ml)	Final Endotoxin Concentration (EU/ml)	Vortex Time (Min)
Standard 1	0.20	---	1.80	1.00	2
Standard 2	---	1.00	1.00	0.50	1
Standard 3	---	0.50	1.50	0.25	1
Standard 4	---	0.20	1.80	0.10	1
Blank	---	---	0.50	0	---

Table 2. Dilutions and procedures for preparing Low Standards (0.01-0.1 EU/mL)

Vial	Volume of Endotoxin Standard Solution (ml)	Volume of Stock	Volume of Standard 1 (ml)	Endotoxin-Free Water (ml)	Final Endotoxin Concentration (EU/ml)	Vortex Time (Min)
Stock	0.20	---	---	1.80	1.00	2
Standard 1	---	0.20	---	1.80	0.100	2
Standard 2	---	---	1.00	1.00	0.050	1
Standard 3	---	---	0.50	1.50	0.025	1
Standard 4	---	---	0.20	1.80	0.010	1
Blank	---	---	---	0.50	0	---

Reconstitute Lyophilized Amebocyte Lysate

- Reconstitute Lyophilized Amebocyte Lysate immediately before use with 1.7 mL of Endotoxin-Free Water (EFW) and swirl gently to dissolve the powder. If more than 1 vial is required, pool 2 or more vials before use. Avoid foaming; do not vortex the solution.

Note: Make sure to recover completely the powder from the sides and the cap of the vial by gently inverting end-over-end. Extreme care must be taken not to touch the inside part of the cap to avoid contamination.

Note: Reconstituted amebocyte lysate solution is stable for 1 week at -20°C or colder, if frozen immediately after reconstitution. Upon thawing, the reconstituted lysate solution may be used only 1 time. Once thawed, gently swirl the reagent to mix before use.

Chromogenic Substrate

Each vial contains 3.4 mg of lyophilized Chromogenic Substrate. Reconstitute the substrate by adding 3.4 mL of Endotoxin-Free Water. Note: Reconstituted Chromogenic Substrate is stable for 4 weeks when stored at 2-8°C. Pre-warm a sufficient substrate amount for the assay to 37°C for no more than 5-10 minutes prior to use

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4.5. ASSAY PROCEDURE

Note: Equilibrate all reagents to room temperature before use. Ensure pipetting order and rate of reagent addition remain consistent from well-to-well and row-to-row throughout the procedure.

- Prepare all reagents and standards as directed in previous section immediately before use.
- Pre-equilibrate plate in a heating block at 37±1°C. Throughout the assay procedure, maintain the plate at 37±1°C.
- Add 50 µL of Endotoxin Standard dilutions, blank, and samples (see "SAMPLE PREPARATION" section) per well.

Note: It is recommended to run each sample and standards in triplicate, including triplicate of a blank (50 µL of Endotoxin-Free Water). *In this case (lot of experimental conditions) samples and Standards are run in duplicate.*

- Keeping the plate at 37±1°C, add 50 µL of the reconstituted Amebocyte Lysate Reagent per well. Begin timing as the lysate is added to the first well.
- Once the Amebocyte Lysate Reagent has been added to the plate wells, briefly remove from the plate heater and mix by gently tapping 10 times on the side of the plate, avoiding spilling. Return the plate to the plate heater and incubate at 37±1°C for the time T1 indicated on the lysate vial.

Note: T1 High = Time 1 for High Standards and T1 Low = Time 1 for Low Standards.

- Reconstitute the Chromogenic Substrate as described in Material Preparation with 3.4 mL of Endotoxin-Free Water. Mix gently by tilting and swirling the vial. Pre-warm to 37±1°C for 5 minutes before use.
- After exactly time T1, add 100 µL per well of pre-warmed reconstituted Chromogenic Substrate Solution.
- Once the substrate solution has been added into all plate wells, briefly remove from the plate heater and mix gently by tapping 10 times to facilitate mixing. Return to the plate heater at 37±1°C for T2 = 6 minutes.
- At exactly T2 = 6 minutes, add 50 µL per well of Stop Solution (25% acetic acid).
- Once the stop solution has been added to the plate wells, remove the plate from the plate heater and mix by gently tapping 10 times on the side of the plate.
- Read the optical density (OD) at 405 nm immediately after assay completion. If the plate is read at a later time, keep covered to avoid evaporation.
- Subtract the average absorbance of the blank replicates from the average absorbance of all individual standards and sample replicates to calculate mean Δ absorbance.
- Prepare a standard curve by pipetting the average blank-corrected absorbance for each standard on the y-axis vs. the corresponding endotoxin concentration in EU/mL on the x-axis. The coefficient of determination, r2, must be ≥0.98.

Note: Do not include the blank OD in the calculation of the regression line.

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- Use the formulated standard curve (linear regression) to determine the endotoxin concentration of each sample.

Table 3. Example data and standard curve for Low Standard.

UE/ml	Avg. OD (405nm)	Δ	St. dev	% CV
0.1	0.5120	0.3937	0.0137	3
0.05	0.3129	0.1945	0.0015	1
0.025	0.1851	0.0668	0.0001	0
0.010	0.1353	0.0170	0.0003	2
0	0.1183	0	0.0024	---

Table 4. Example data and standard curve for High Standard.

UE/ml	Avg. OD (405nm)	Δ	St. dev	% CV
1	1.4327	1.3162	0.0774	6
0.5	0.7382	0.6217	0.0158	3
0.25	0.3388	0.2223	0.0045	2
0.10	0.1581	0.0416	0.0018	4
0	0.1165	0	0.0033	---

4.6. SAMPLE PREPARATION

4.6.1. ENM SAMPLE DISPERSION PREPARATION

Prepare ENMs according to the protocol agreed within WP4-5-6 with the following precautions:

- Use a pyrogen free glass vial to weight ENMs and disperse them in endotoxin free water (provided with the kit).
- Test a proper concentration of ENMs. The highest concentration used in the toxicology tests might be considered (e.g. 100 ug/ml. To test this, prepare a ENM concentration of 200 ug/ml). Lower concentrations might be used to avoid interference of ENMs with the test.
- Check the pH of the ENMs dispersion and adjust it if needed with pyrogen free NaOH: the pH should be between 6 and 8, otherwise interference can be expected. However, changes in pH and ionic strength might affect ENM physicochemical properties.

Samples preparation for interference test

1. To verify the lack of interference, spike the ENM dispersion with a known amount of endotoxin (e.g., 0.5 EU/mL). Assay the spiked sample and the un-spiked samples to determine the respective endotoxin concentrations. The difference between the two calculated endotoxin values should equal the known concentration of the spike ±25%.

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- Mix the ENM dispersion 1:1 with endotoxin solution (e.g. mix the 200 ug/ml ENM concentration, with the endotoxin Standard 1 (0.1 EU/ml), in order to obtain a final dispersion of 100 ug/ml ENM and a 0.05 EU/ml endotoxin concentration (corresponding to the Standard 2)).
- To determine if a sample's intrinsic color will alter the absorbance readings, construct a mock reaction tube by adding 50µL of sample, 150µL of endotoxin-free water and 50µL of Stop Reagent with no incubation. Read the absorbance at 405-410nm. If the absorbance is significantly greater than the absorbance of endotoxin-free water, then the intrinsic color will alter the correct sample absorbance readings. In such cases, include appropriate controls in the assay.Read the absorbance at the same wavelength of the test (405 nm), and record the absorbance spectrum of the samples between 300 and 600 nm.

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