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Endo-RS®

Endotoxin Recovery Kit (Surfactant)

Toolbox for demasking endotoxin in biopharmaceutical formulations containing surface active agents

Package Insert Endo-RS®

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1. General Information

1.1 Intended Use

Intended use

The Endo-RS® Endotoxin Recovery Kit provides reagents for demasking endotoxins in biopharmaceutical drug formulations which are not detectable in commonly used testing procedures (*e.g.* the Limulus amebocyte lysate, LAL test).
The Endo-RS® kit is intended for samples in which masking is mainly triggered by non-ionic surface active agents (surfactants) such as Polysorbate 20, Polysorbate 80 or Triton X-100 and not by proteins.
The Endo-RS® Kit is intended for sample preparation of biopharmaceutical drug formulations with subsequent endotoxin detection using the EndoLISA® Endotoxin Detection Assay.

1.2 Scientific Background and Test Principal

Endotoxin

Endotoxins are bacterial cell membrane constituents which are recognized by the human immune system and trigger severe physiological reactions. Chemically, endotoxins are lipopolysaccharide (LPS) originating from gram-negative bacteria. LPS is composed of a conserved part (lipid A + conserved core carbohydrate structure) and a highly variable part (O-antigen).

Endotoxin Masking (also referred to as Low Endotoxin Recovery, LER)

The non-detectability of endotoxin within commercially available endotoxin test systems is a commonly known phenomenon [1]. Thereby, the non-detectability can be caused by either test interference or the masking of endotoxin. In the first case, interference is caused by ingredients in the sample which influence the activity of the enzymes of the Limulus Amebocyte Lysate (LAL). Thus, the Positive Product Controls (PPC) are invalid. In this case, interference can be overcome by using a more robust test system like EndoLISA or by diluting out the interfering substances.

In the case of endotoxin masking, the ingredients of a sample have a direct impact on the structure of the endotoxin. Lipopolysaccharides are amphiphile and tend to self-assembly in aqueous solutions. Thus, under certain circumstances the aggregation state of LPS can convert from a highly LAL-reactive state to a less or non-active state. In contrast to the interference case, this process is time and temperature dependent.

Endotoxin masking is described in literature for several proteins [2] and blood products [3]. Recent studies have shown that masking is not only be caused by proteins but can also be triggered by surfactants in a sample [4]. Within such samples, masking is mainly triggered by two reasons. First, the LPS aggregates are destabilized by chelating or competing out bivalent cations and second, by incorporation of LPS monomers into surfactant micelles (LPS-Masker complex). These embedded LPS monomers are non-detectable because of two reasons. The binding site of the Lipid A part of the LPS for the enzymes of the LAL reagent are not accessible anymore and monomeric LPS molecules are not active in limulus based detection methods [5].

Demasking of Endotoxin

For demasking, the LPS monomers have to be liberated from the LPS-Masker complex and a LAL- reactive structure has to be reconfigured. Masking and the stability of the LPS-masker complex strongly depend on the masking ingredients and concentrations. Thus, a suitable demasking protocol for a certain product formulation has to be developed.

To this end, the Endo-RS® Endotoxin Recovery Kit provides you with a toolbox of reagents for demasking samples in which masking is mainly triggered by surfactant and not by proteins. The kit includes a component which is able to disturb stability of the LPS-masker complex (Disturber; Component B), a component which is able to adsorb surfactant (Adsorber; Component C), two different kinds of modulators (Component D1 and D2) which support liberation and presentation of LPS to the Reconfigurator component (E). The latter component is essential to induce formation of detectable LPS structures.

2. Kit Components and Safety Information

Kit components

Component	Container	Content	Description
A Buffer (**B**)	Plastic bottle, transparent cap	1 x 3.85 mL	Buffer for pH adjustment of samples
B Disturber* (**DIST**)	Plastic bottle, white cap	1 x 2.6 mL	Agent for destabilization of LPS-Masker-Complex. This kit component contains calcium chloride. Please read safety information below.
C Adsorber (**ADS**)	Glass bottle, aluminium cap	1 bottle	Agent for surfactant adsorption, lyophilized. This kit component contains products of animal origin. (Bovine Serum Albumin)
D1 Modulator 1 (**MOD1**)	Plastic bottle, brown cap	1 x 2.6 mL	Agent for supporting Reconfigurator component
D2 Modulator 2* (**MOD2**)	Plastic bottle, red cap	1 x 2.6 mL	Agent for supporting Reconfigurator component. This kit component contains Sodium Dodecyl Sulfate. Please read safety information below
E Reconfigurator** (**REC**)	Plastic bottle, green cap	1 x 2.6 mL	Agent for LPS aggregate structure formation. This kit component contains ethanol. Please read safety information below.
F Endotoxin Standard (**CSE**)	Glass bottle, yellow cap	1 bottle	LPS from *E. coli* O55:B5, lyophilized, matrix-free
G Water (**WEF**)	Plastic bottle, blue cap	1 x 30 mL	Endotoxin-free water for reconstitution of endotoxin and adsorber and dilution of components and samples

(*)SIGNAL WORD : **WARNING**

H319
P280 / P305+P351+P338 / P337+P313
Hazard statement:
H319 : Causes serious eye irritation.
Precautionary statement:
P280 : Wear protective gloves/protective clothing/eye protection/face protection.
P305+P351+P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337+P313 : If eye irritation persists: Get medical advice/attention.
For further information, refer to the Material Safety Data Sheet.

(**)SIGNAL WORD : **DANGER**

H225 / H319
P210 / P280 / P305+P351+P338 / P337+P313 / P403+P235
Hazard statement:
H225 : Highly flammable liquid and vapour.
H319 : Causes serious eye irritation.
Precautionary statement:
P210 : Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking..
P280 : Wear protective gloves/protective clothing/eye protection/face protection..
P305+P351+P338 : IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337+P313 : If eye irritation persists: Get medical advice/attention.
P403+P235 : Store in a well-ventilated place. Keep cool.
For further information, refer to the Material Safety Data Sheet.

3. Warnings and Precautions

Warning:

The Endo-RS® Kit is not intended for samples without surfactants in which endotoxin masking is solely triggered by proteins.
For professional use only.
The kit contains products of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not totally guarantee the absence of transmissible pathogenic agents. It is therefore recommended that these products be treated as potentially infectious, and handled observing the usual safety precautions (do not ingest; do not inhale).
Due to interference of the high content of added demasking reagents with LAL reagents, the demasked endotoxin has to be detected using the EndoLISA® Endotoxin Detection Assay.
The reagents in the kit provide you with a toolbox for demasking endotoxins. Due to differences in buffer compositions of masked samples, the amounts and concentrations of the required demasking reagents must be adapted for each individual formulation in order to optimize the endotoxin recovery.

Endotoxin-free conditions

All materials used, such as containers or pipette tips, should be purchased endotoxin-free. For preparing samples, glass test tubes (e.g. EndoGrade® Glass Test Tubes) are recommended, since endotoxin may adhere to hydrophobic plastic surfaces.

Treatment of glass materials

After standard cleaning procedure, glass should be "baked" at +200°C for 4h. Use aluminum caps or aluminum foil to seal openings.

Treatment of plastic materials

Plastic material may be treated with 1 M NaOH for 6-12 h. Afterwards rinse with a large volume of endotoxin-free water and let it air dry. Final pH of the rinsing water should be neutral.

Handling of sample material

Samples should be stored refrigerated or frozen. Treat samples carefully in order to avoid microbial or endotoxin contamination. All materials in direct contact with the sample or kit reagents must be endotoxin-free.

4. Additional Reagents, Equipment, Instrumentation and Software Required

Reagents

- EndoLISA® Endotoxin Detection Assay and required instrumentation for the quantification of the endotoxin content (Ref. 609033, 192 tests).
- Ethanol (70%), endotoxin-free
- EndoGrade® Water, 30 mL (Cat. No. 607030, 20 bottles/pack): Endotoxin-free water for sample and standard dilutions
- EndoGrade® Water, 100 mL (Cat. No. 607100, 16 bottles/pack): Endotoxin-free water for sample and standard dilutions
- EndoGrade® Water, 500 mL (Cat. No. 607500, 6 bottles/pack): Endotoxin-free water for sample and standard dilutions

Equipment

- Pipettes
- Endotoxin-free pipette tips
- Endotoxin-free glass test tubes (EndoGrade® Glass Test Tubes, Ref. 800050)

Instrumentation

Vortex-type mixer
A multi-tube vortex-type mixer is recommended (e.g. Heidolph Multi Reax test tube shaker).

5. Reagent Storage and Preparation

Storage and stability

Unopened kits are stable at + 2 to + 8°C until the expiry date printed on the label. For further information on storage and stability of the individual components, please refer to the table below.

Once a vial has been opened, it should be stored at the temperature indicated in the table below for no longer than four (4) weeks. All components should be brought to room temperature before use.

Use of kit components / storage conditions

Reagent	Preparation of working solutions	Storage conditions of working solutions
A Buffer (**B**)	Ready-to-use	+2 to +8°C
B Disturber (**DIST**)	Ready-to-use	+2 to +8°C
C Adsorber (**ADS**)	Reconstitute in 2.6 mL water G	-20°C.
D1 Modulator 1 (**MOD1**)	Ready-to-use	+2 to +8°C
D2 Modulator 2 (**MOD2**)	Ready-to-use	+2 to +8°C
E Reconfigurator (**REG**)	Ready-to-use	+2 to +8°C
F Endotoxin Standard (**CSE**)	Reconstitute with the imprinted volume of water G to obtain a approx. 10.000 EU/mL solution of LPS from *E. coli* O55:B5	Stable for 4 weeks when stored at +2 to +8°C or until expiry date of the kit when stored frozen in aliquots at -20°C. Freeze and thaw only once.
G Water (**WEF**)	Ready-to-use	Stable until expiry date of the kit when stored at +2 to +8°C

6. Endotoxin Recovery Procedure

Overview general experimental workflow:

The Endo-RS® kit is intended for samples in which endotoxin is masked in commonly used formulation compositions of biopharmaceuticals. Affected compositions typically contain phosphate or citrate buffer with pH between 5.5-8.0 and non-ionic surfactants just above their theoretical critical micellar concentrations (CMC) (example: approx. 0.05 wt% of Polysorbate 20 or Polysorbate 80 or Triton X-100).

6.1 General Handling Instructions

Handling instructions

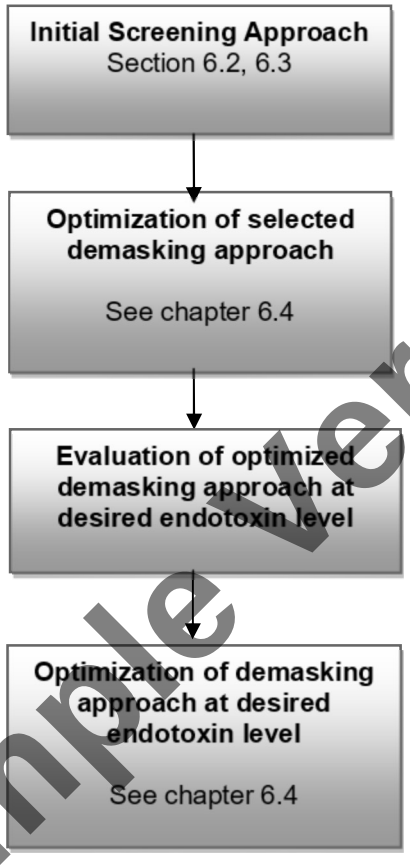
- Be careful not to contaminate the kit components in use.
- Let all reagents reach room temperature (+20 to +25°C) before use.
- Pipette carefully to ensure accurate transfer of the small volumes.
- Perform all preparations in duplicates.
- Reagents from different lots **MUST NOT** be mixed.

6.2 General Demasking Procedure

Prerequisites:

The Endo-RS® kit provides a toolbox containing several components for demasking the endotoxin in affected samples. Due to the presence of further composition ingredients in the masked samples, it is necessary to select the appropriate components and to adapt the amounts/concentrations of the demasking components to obtain optimal endotoxin recoveries.

The development of an optimal demasking protocol is an iterative process, consisting of an initial screening for the best suited demasking approach and a subsequent optimization of component concentrations.



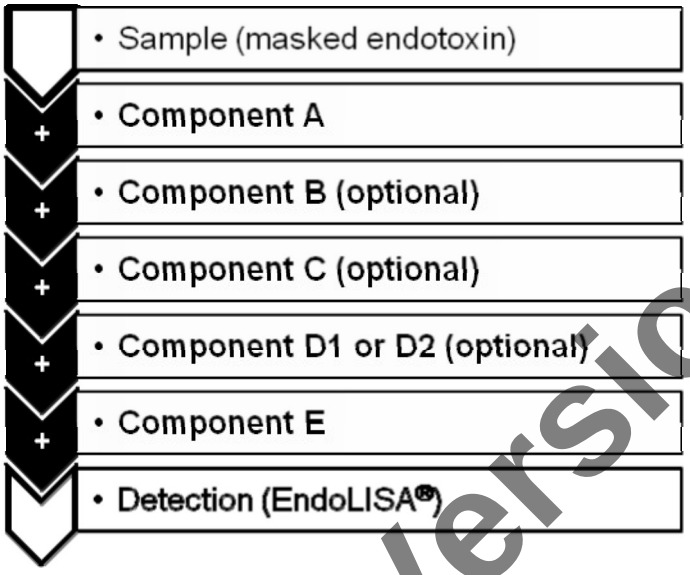
Initial screening approach:

The following table depicts four recommended demasking approaches of the initial screening:

Demasking approach #	A Buffer	B Disturber	C Adsorber	D1 Modulator 1	D2 Modulator 2	E Reconfigurator
1	+	-	-	-	-	+
2	+	-	+	-	-	+
3	+	-	+	+	-	+
4	+	+	+	-	+	+

As the concentration of the Reconfigurator (Component E) is most critical during demasking, the initial screening should be performed depending on Reconfigurator (Component E) concentration.

The required components for demasking (Component A, B, C, D1, D2 and E) should be added sequentially in alphabetical order (as indicated) including a two minutes mixing step after each addition of a component:



6.3 Detailed Description of the Initial Screening Step

General instructions

- Samples must be prepared using depyrogenated equipment and endotoxin-free reagents.
- Before use, adjust all reagents and samples to room temperature.

6.3.1 Endotoxin Masking

Prerequisites for endotoxin masking

Prior to starting demasking experiments, it is recommended to prepare masked endotoxin in your sample. Endotoxin spikes of 100 EU/mL in the sample to be tested are recommended. After development of a successful demasking protocol, the sensitivity limit for the demasking protocol should be determined by analyzing samples containing different endotoxin concentrations. A final optimization of the protocol at low levels of endotoxins is recommended to improve endotoxin recovery.

Procedure endotoxin masking

- The endotoxin (Component F) is matrix free! Thus, the glass vial appears to be empty. Remove the rubber cap slowly and carefully.Reconstitute endotoxin (Component F) for masking and demasking in the volume of endotoxin-free water (bottle G) imprinted on the label and vortex for at least 10 minutes. The endotoxin stock solution has an endotoxin content of approximately 10,000 EU/mL.
- Prepare 13 samples with a volume of 1 mL containing 100 EU/mL of the endotoxin stock solution in endotoxin-free glass test tubes (add 10 µL of the endotoxin stock solution to 990 µL of your sample).
- Prepare one additional endotoxin control sample (1mL) in endotoxin-free water (bottle G)in endotoxin-free test tubes containing the same amount of endotoxin as your masked samples (#14 in the table on the next page).
- Mask added endotoxin in the samples by incubation.

Important:

Masking strongly depends on incubation time, temperature and formulation. Thus, masking conditions should be evaluated before starting demasking in separate masking monitoring experiments. Thereby, masking kinetics of the drug product as well as of the formulation (placebo) indicate the masking capacity of the sample. (a detailed guide regarding masking monitoring experiments is available from www.hyglos.com).