BIOMÉRIEUX

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ENDOZYME® II GO

Fast Recombinant Factor C Endotoxin Detection Assay

Fluorescence microplate assay using the high-speed GOPLATE™ prefilled with Control Standard Endotoxin (CSE) and Recombinant Horseshoe Crab Factor C (rFC).

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1. General Information

1.1 Intended Use

ENDOZYME® II GO is intended for quantitative determination of endotoxin (chemically lipopolysaccharide, LPS) in liquid samples such as water. It can also be used for quantitative determination of endotoxin in pharmaceutical final products, in-process control and medical device testing.

1.2 Test Principle

ENDOZYME® II GO is a homogeneous enzymatic assay which uses the synthetic endotoxin receptor (recombinant Factor C) derived from the blood clotting cascade of horseshoe crabs in combination with a fluorogenic substrate.

Endotoxin Endotoxins are bacterial cell membrane constituents which are recognized by the human immune system and may trigger severe physiological reactions. The main endotoxin of Gram-negative bacteria is lipopolysaccharide (LPS). LPS is composed of a conserved part (lipid A + conserved core carbohydrate structure) and a highly variable part (O-antigen).

Limulus coagulation cascade In blood cells of horseshoe crabs such as *Limulus polyphemus* and *Tachypleus tridentatus*, the amebocytes, a coagulation cascade has evolved to resist infections caused by Gram-negative bacteria. The principal receptor of this proteolytic cascade is a protein named Factor C. It is a zymogen/proenzyme (precursor of an enzyme, here protease) that is activated by endotoxin.

Recombinant Factor C (rFC) Recombinant Factor C (rFC), instead of *Limulus* or *Tachypleus* amebocyte lysate (LAL or TAL), is used in combination with a synthetic fluorogenic substrate for detection of endotoxin.

1.3 Specifications

Assay range 0.005 to 50 EU/mL

EU = Endotoxin Unit / 1 EU = 1 IU

1 EU corresponds to 0.1 ng LPS (Food and Drug Administration (FDA) Reference standard endotoxin (RSE) *E. coli* O113 EC-7)

Quantitation limit 0.005 EU/mL

Assay time 60 minutes (0.005 EU/mL)

A shorter assay time is possible depending on required quantitation limit (sensitivity) (see section 6.4)

2. Kit Components

Number of tests The kit contains reagents for 192 tests.

Kit Component	Container	Content	Description
1 Enzyme **(ENZ)**	Plastic bottle, transparent cap	1 x 2.5 mL	Enzyme (rFC) solution. This kit component contains products of animal origin (Bovine Serum Albumin).
2 Substrate (**SUB**)	Brown plastic bottle, brown cap	1 x 2.5 mL	Fluorescence substrate.
3 GOPLATE™	Plastic bag	2 plates	Ready-to-use assay plates pre-loaded with Control Standard Endotoxin (CSE) of *E. coli* O113:H10; standard curve concentrations and Positive Product Controls (PPCs).
4 Water (**WEF**)	Plastic bottle, blue cap	2 x 100 mL	Water, free of detectable levels of endotoxin and non-interfering, for addition to wells containing standard concentrations and blanks as well as for dilution of samples.
5 Assay Buffer (**AB**)	Brown plastic bottle, brown cap	2 x 12 mL	Assay Buffer, to be combined with Substrate 2 and Enzyme 1.

1 Package Insert downloadable from www.biomerieux.com/techlib

3. Warnings and Precautions

Warning:

ENDOZYME® II GO is not intended for use with clinical samples or for diagnosis of human or animal disease.
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).
Each GOPLATE™ is for single use and should not be reused.

Endotoxin-free conditions

All materials used, such as containers or pipette tips, should be free of detectable levels of endotoxin and interference. For preparing sample, glass test tubes are recommended, since endotoxin may adhere to hydrophobic plastic surfaces.

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 test reagents must be free of detectable levels of endotoxin and without interference.

4. Additional Reagents, Equipment, Instrumentation and Software Required

Equipment required

- Pipettes
- Multi channel pipette or dispensing pipette
- Pipette tips, endotoxin-free and without interference
- Glass test tubes, endotoxin-free (e.g. ENDOGRADE® Glass Test Tubes – Ref. 800050)

Instruments

Vortex-type mixer

0-1500 rpm
Sample dilutions should be mixed vigorously for 1 minute. This is optimally achieved by using a multi-tube Vortex-type mixer.

Incubator (optional)

The incubation of the assay plate should ideally be performed in the fluorescence reader at +37°C. Alternatively the assay plate can be incubated in an incubator at +37°C between measuring time point zero and measuring time point 60 minutes.

Fluorescence microplate reader

Fluorescence microplate readers from different suppliers may be used for reading of ENDOZYME® II GO results.

Instrument settings:	Temperature	+37°C
	Excitation (nm)	380
	Emission (nm)	445*
	Optics position	Top
	Readings per well	Minimum 10
	Shaking mode	On**
	Sensitivity/PMT gain	To be determined for each instrument and reagent lot (see section 6.3)

* 445 is the optimum wavelength. A 440 filter can be used.

** Shake for 15 seconds at medium intensity prior to reading Time Point 0.

Calculation software

For standard curve fitting and back-calculation of the endotoxin content of unknown samples, calculation software is required. ENDOZYME® II GO standard curve is generated by fitting a linear regression function to the logarithmic data of both endotoxin concentration and net dRFU (see section 6.9). Alternatively, a 4-parameter-logistic function can be used to extend the dynamic range to 50 EU/mL (see section 6.10).

5. Reagent Storage and Preparation

Storage and stability

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

Use of kit components, stability and storage conditions

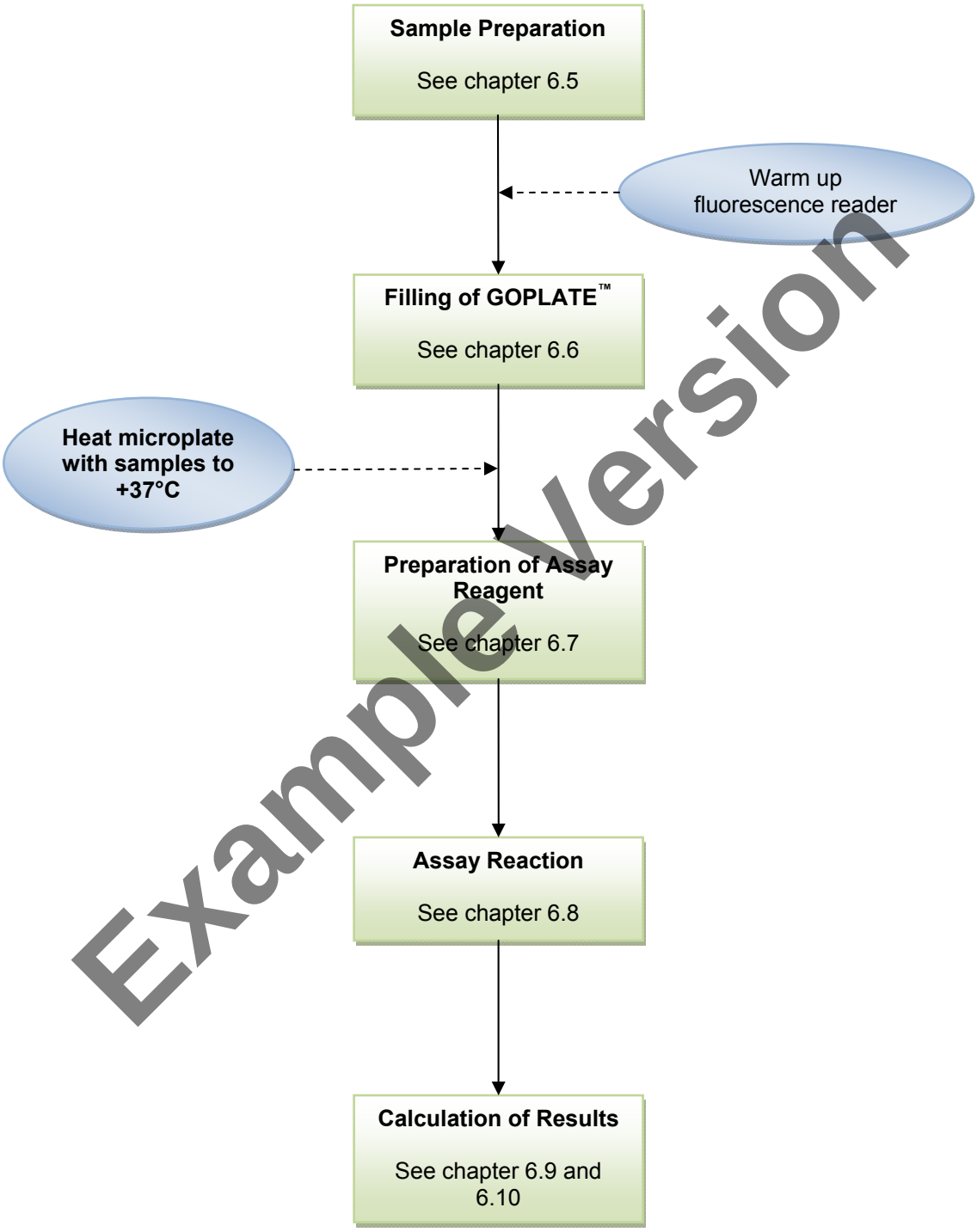
Reagent	Preparation	Stability and storage conditions of working solutions
1 Enzyme (**ENZ**)	For Assay Reagent preparation	Stable until expiry date of the kit when stored between +2°C and +8°C
2 Substrate (**SUB**)	For Assay Reagent preparation	Stable until expiry date of the kit when stored between +2°C and +8°C
3 GOPLATE™	Ready-to-use	Unopened stable until expiry date of the kit. Upon opening for immediate use.
4 Water (**WEF**)	Ready-to use	Stable until expiry date of the kit when stored between +2°C and +8°C
5 Assay Buffer (**AB**)	For Assay Reagent preparation	Stable until expiry date of the kit when stored between +2°C and +8°C

Reagents to be prepared from kit components

Assay Reagent: Prepare the Assay Reagent freshly immediately before use (see chapter 6.7 for quantities).

6. Assay Protocol

6.1 Overview Assay Procedure



6.2 General Handling Instructions

Handling instructions

- All reagents needed for running ENDOZYME® II GO are supplied with the kit.
- Be careful not to contaminate the kit components in use.
- Let all reagents reach room temperature (+20°C/+25°C) before use.
- Pipette thoroughly to ensure accurate transfer of the small volumes.
- Perform all measurements at least in duplicates.
- Reagents from different lots **MUST NOT** be mixed and used in one test series.

6.3 Gain Adjustment

Procedure

Done at the installation of the reader and once per reagent lot.
The logarithm of the dRFU correlates with the logarithm of the gain. Accordingly, the optimum gain can be determined by analyzing several 0.5 EU/mL replicates:
Pipette 100 µL of Water (4) into six PPC wells, e.g. G10, G11, G12, H10, H11 and H12.
Put the microplate into the fluorescence reader and warm up the microplate to +37°C.
Combine 640 µL Assay Buffer (5), 80 µL Substrate (2) and 80 µL Enzyme (1) in an endotoxin-free and non-interfering reagent reservoir or tube.
Add 100 µL assay reagent (see 6.7 Preparation) to each of the previously mentioned six wells.
Run the assay according to section 6.8 Assay Run using the same six different gains both at time point zero and after 60 minutes or final reading time point.

Calculation of the optimum gain

Subtract time point zero data from time point 60 minutes data (dRFU) and calculate the mean dRFU for each gain.
Plot the calibration curve (log(dRFU) vs. log(gain)). Use the mean dRFU of each gain.
Calculate the curve function by fitting a linear model to the data:
log(dRFU) = A*log(gain)+B

Calculate the optimum gain:
optimum gain = 10^((log(optimum dRFU)-B)/A)

- For linear regression the optimum dRFU = 5% of maximum RFU
- For 4-parameter logistic regression the optimum dRFU = 2% of maximum RFU

The optimum gain must be within the range of tested gains. Otherwise, the gain determination should be repeated with a different set of gains.

6.4 Control Standard Endotoxin

Standard concentrations

The GOPLATE™ (3) is pre-filled with the following concentrations of Control Standard Endotoxin from *E. Coli* O113:H10 (plate layout see section 6.6).

	Linear regression model	Non-linear regression model
50 EU/mL	n/a	+
5 EU/mL	+	+
0.5 EU/mL	+	+
0.05 EU/mL	+	+
0.005 EU/mL	+	+

If a sensitivity of, for example, only 0.05 EU/mL is required, the assay run time can be shortened and 0.005 EU/mL are excluded from the analysis. This has to be validated by the user.

6.5 Sample Preparation

Sample preparation/ sample dilution

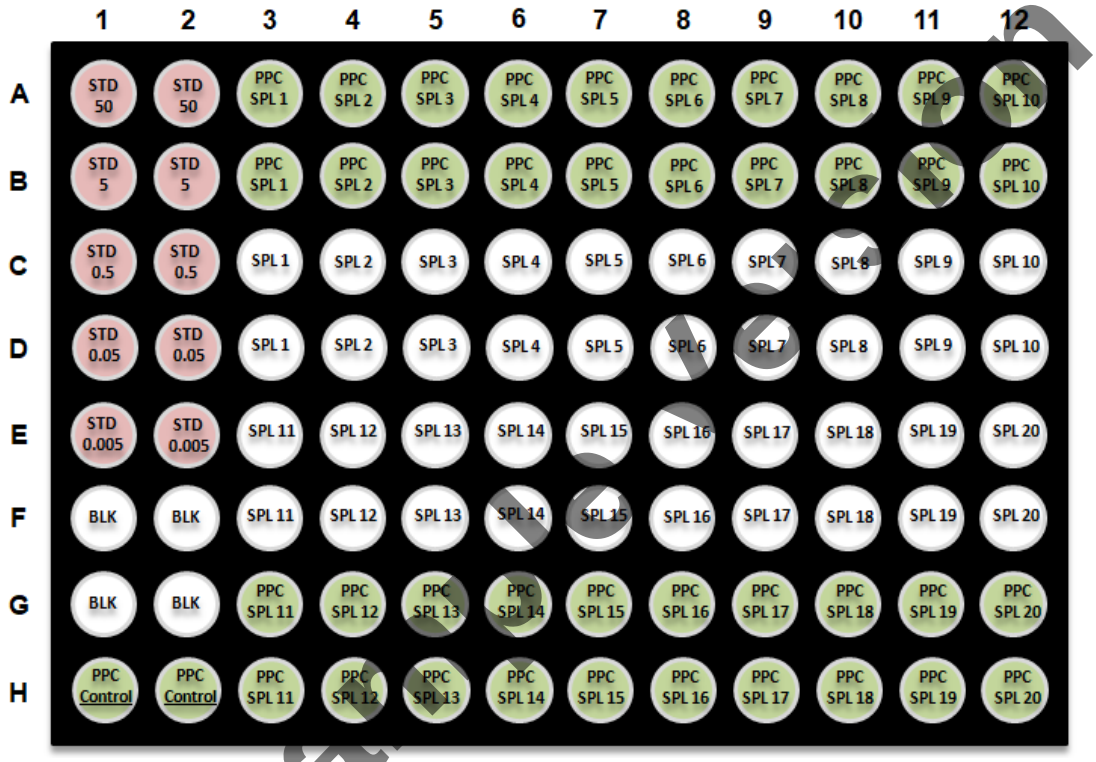
- For sample dilution, use endotoxin-free and non-interfering glass test tubes.

  For example, a 1:10 dilution should be prepared as follows:

- Pipette 900 µL of Water (4) into a vial and add 100 µL of sample. Mix using a vortex-type mixer for at least 1 minute.

6.6 Filling of the GOPLATE™

GOPLATE™ layout The GOPLATE™ is pre-loaded with Control Standard Endotoxin (CSE) as follows:



STD = Control Standard Endotoxin (CSE), e.g. STD 50 = 50 EU/ mL
BLK = blank, empty well
PPC = Positive Product Control (= 0.5 EU/mL)
SPL = sample, empty well

Filling of the GOPLATE™ Reconstitute CSE and PPC control and add blank by pipetting 100 µL of Water (4) into each well of columns 1 & 2.
Pipette 100 µL of sample into at least four wells: two wells without PPC, two wells with PPC (see GOPLATE™ layout above). Put the microplate into the fluorescence reader and warm up the microplate with samples to +37°C.

6.7 Preparation of Assay Reagent

Assay Reagent mix Required amounts are indicated in the table below.
Combine 8 parts of Assay Buffer (5), 1 part of Substrate (2) and 1 part of Enzyme (1).
Mix gently - **do not mix with a vortex-type mixer.**

Apply the indicated volumes in an endotoxin-free and non-interfering reagent reservoir or tube:

Assay Reagent	Assay Buffer	Substrate	Enzyme
4 mL for 32 reactions	3.2 mL	0.4 mL	0.4 mL
6 mL for 48 reactions	4.8 mL	0.6 mL	0.6 mL
8 mL for 64 reactions	6.4 mL	0.8 mL	0.8 mL
10 mL for 80 reactions	8.0 mL	1.0 mL	1.0 mL
12 mL for 96 reactions	9.6 mL	1.2 mL	1.2 mL

6.8 Assay Run

Detection step

- Add 100 µL of Assay Reagent to each well.

  Recommended: Use a dispensing pipette or a multi-channel pipette in order to reduce the hands-on time.

- Close the reader containing the plate and wait 1 min to allow the temperature to adjust.
- Shake for 15 seconds at medium intensity.
- Read fluorescence signals at time point zero (first reading).
- Incubate the microplate for the appropriate time depending on the sensibility required (60 minutes or less) at +37°C (incubator or fluorescence reader).
- Read fluorescence signals (second reading).

6.9 Calculation of Results: Linear Regression Model of Standard Curve

Blank correction Subtract time point zero data from time point 60 minutes data (dRFU).
Calculate the mean dRFU of the blank.
Subtract the mean blank dRFU from the dRFU of standards and samples (net dRFU).

Standard curve Plot the standard curve (log(EU/mL) vs. log(net dRFU)). Use the mean net dRFU of each CSE concentration in the range 0.005-5 EU/mL.
Calculate curve function by fitting a linear model to the data:

$$\log(dRFU) = A*\log(EU/mL)+B$$

Calculate the correlation coefficient (|r| should be ≥ 0.980).

Sample values Calculate endotoxin concentration (EU/mL) of samples using the linear function.
Multiply results with the dilution factors of the samples.

Spike Recovery Calculate the endotoxin concentration difference between – if necessary, diluted – sample and respective Positive Product Control. Determine, if it is within 50% to 200% of the expected value, e.g. 0.25 to 1 EU/mL for a spike of 0.5 EU/mL.

6.10 Calculation of Results: 4-Parameter Logistic Regression Model of Standard Curve

Rationale

To extend the measurement range by one order of magnitude, a standard at 50 EU/mL is included. However, it is beyond the linear range of the assay. In this case, a non-linear regression model should be fitted to the data.

Data handling

Subtract time point zero values from 60 minute values (dRFU).
Calculate standard curve according to the following equation:

dRFU = (A-D)/(1+(EU/mL/C)^B)+D
fit weight: 1/dRFU

Calculate endotoxin concentration (EU/mL) of samples using the non-linear function.
Calculate the correlation coefficient (|r| should be ≥ 0.980).

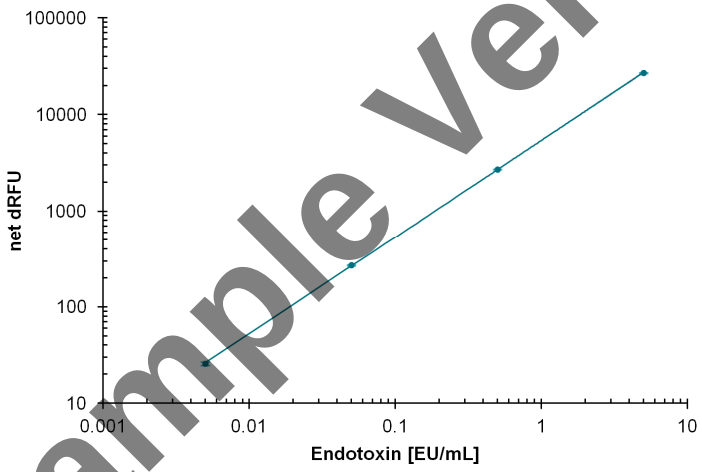
Spike Recovery

Calculate the endotoxin concentration difference between – if necessary, diluted – sample and respective PPC. Determine, if it is within 50% to 200% of the expected value, e.g. 0.25 to 1 EU/mL for a spike of 0.5 EU/mL.

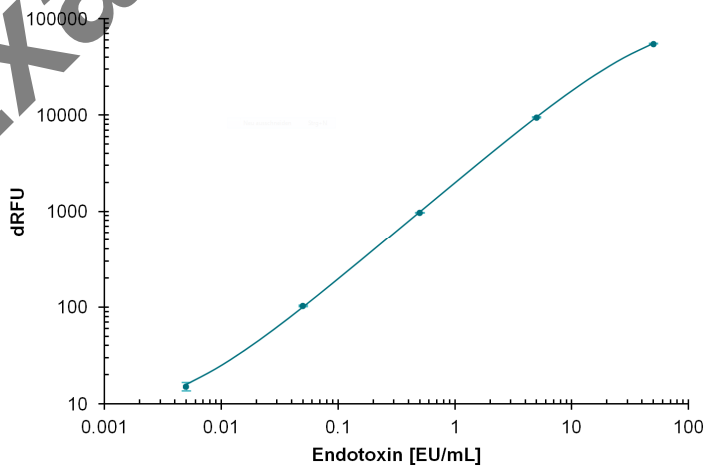
6.11 Standard Curve Examples

Note: Values may differ depending on the instrument sensitivity gain.

Linear regression



4-parameter logistic regression



6.12 Influencing Parameters and Limitations

Test interference can cause invalid results, as revealed by invalid spike recovery due to alteration of the enzymatic reaction conditions. Usually, such interference can be overcome by sample dilution in endotoxin-free water. The maximum valid dilution factor (MVD) is calculated using the formula below, where endotoxin limit is the maximum acceptable endotoxin concentration in the undiluted sample and assay sensitivity is the lowest standard concentration, e.g. 0.005 EU/mL for ENDOZYME® II GO:

MVD = Endotoxin limit / Assay sensitivity

If interference of a sample cannot be overcome by valid dilution or other sample treatment, this test method is not suitable. In those cases, we recommend using the ENDOLISA® Endotoxin Detection Assay.

Another phenomenon distorting endotoxin testing is endotoxin masking known as Low Endotoxin Recovery (LER). Lipopolysaccharides are amphiphilic and tend to aggregate in aqueous solutions. Under certain circumstances, the aggregation state of LPS can convert from a highly Factor C-activating state to a less or non-activating state. In case of LER, sample ingredients demonstrate such a direct impact on the structure of endotoxin. In contrast to test interference, LER is time-dependent and dilution-independent. For samples showing LER, the ENDO-RS® Endotoxin Recovery Kit can demask endotoxin which can be detected with the ENDOLISA® assay, i.e. to allow valid time-independent endotoxin recovery.

A range of potential test-influencing parameters are listed below:

Temperature	For the detection reaction, +37°C is the optimum. Before use, the assay components should be adjusted to room temperature.
Agitation	After addition of the assay mixture, the plate should be shaken thoroughly.
pH	Samples with extreme pH values may influence assay performance, if the buffer capacity of the test system is exhausted. Dilution or pH adjustment to pH 7 is recommended.
Salt	Total salt concentration in a sample should not exceed 500 mM. Otherwise, dilution is required.
Detergents	Detergents may interfere with ENDOZYME® II GO. Dilution is recommended. By forming mixed aggregates with endotoxin, they may stimulate LER.
Chelating agents	Chelating agents (e.g. EDTA, EGTA, and citrate) absorb cations. If such agents are present, dilution or neutralization of the chelating agent, e.g. with magnesium, is recommended. By destabilizing endotoxin, chelating agents may stimulate LER.
Chaotropic agents	Chaotropic agents may modulate hydrophobic interactions and denature Factor C. Dilution is recommended. ENDOLISA® is very robust against chaotropic agents.
Organic solvents	Organic solvents may modulate hydrophobic interactions and denature Factor C. Dilution is recommended. ENDOLISA® is very robust against organic solvents.
Proteins	Protein interference strongly depends on the physical and chemical properties of the proteins. Dilution is recommended. ENDOLISA® is very robust against protein interference. Proteins may stimulate LER by absorbing endotoxin.
Proteases Peptidases	Serine proteases/peptidases like trypsin may mimic Factor C's activity and cause false-positive results, i.e. fluorescence development in the absence of endotoxin. Proteases can be revealed by recording the reaction kinetics of ENDOZYME® II GO, i.e. read fluorescence several times throughout the assay runtime. The reaction curve of Factor C is parabolic, while other proteases usually show linear reaction kinetics. In this case, heat treatment at +75°C for 15 min is recommended. Alternatively, protease inhibitors can be used in combination with ENDOLISA® (avoid EDTA).
Blood products	ENDOZYME® II GO is generally not suitable for the direct detection of endotoxin in serum, plasma or blood samples.

7. Waste Disposal

Unused reagents may be considered as non-hazardous waste and disposed of accordingly. Dispose of used reagents as well as any other contaminated disposable materials following procedures for infectious or potentially infectious products. It is the responsibility of each laboratory to handle waste and effluents produced according to their nature and degree of hazardousness and to treat and dispose of them (or have them treated and disposed of) in accordance with any applicable regulations.

8. Quality Control

ENDOZYME®II GO has been designed and developed to meet the strictest quality requirements. The results of quality control are given on the quality control certificate available from our website (www.biomerieux.com).

9. Troubleshooting Guide

Observation	Possible Cause	Measure
No signal at all	▪ Inappropriate instrument settings	⇒ Check instrument parameter
	▪ Lamp defect	⇒ Change lamp
	▪ Pipetting error	⇒ Check reagents, repeat assay
	▪ Incubation temperature much too high or much too low	⇒ Check temperature setting
No signal with individual samples	▪ Pipetting error (no water or sample pipetted)	⇒ Repeat assay
	▪ Interfering ingredients	⇒ Check spike control; dilute sample
	▪ Inappropriate pH	⇒ Check pH; neutralize sample
Low signal level	▪ Instrument sensitivity (gain) too low	⇒ Enhance sensitivity; higher gain needed
	▪ Reader defect (e.g. optics)	⇒ Run instrument check
	▪ Incubation temperature too high/too low	⇒ Check temperature
	▪ Kit damage (shipment or storage)	⇒ Check storage conditions and package material; contact technical service
	▪ Kit or working solutions expired	⇒ Use new kit or fresh reagents
	▪ Inappropriate emission wavelength or band	⇒ Emission should be measured around 445 nm; band 20-40 nm
High background signal in standards and negative control	▪ LPS contamination of assay components (e.g. water)	⇒ Use fresh reagents
	▪ LPS contamination of vials or pipette tips	⇒ Use different lot of vials and pipette tips; switch to glass vials or change supplier
	▪ Inappropriate excitation wavelength or band	⇒ Excitation should not be below 360 nm, band 10-20 nm
High well-to-well variation	▪ Temperature gradient (incubator, reader)	⇒ Change incubator, reader
	▪ Pipetting error	⇒ Calibrate pipettes
Invalid spike control	▪ Interfering ingredients	⇒ Dilute sample
	▪ Inappropriate pH	⇒ Check pH; neutralize sample
Non-sigmoidal 4-parameter logistic standard curve	▪ Negative dRFU in blank (dependent on instrument)	⇒ Use linear regression model (up to 5 EU/mL)
		⇒ Use different reader model

10. Legal Statements and Regulatory Information

Validation and Regulatory Information

Recombinant Factor C is included as an alternative method in the European Pharmacopoeia (Ph. Eur.) chapter 5.1.10, Section 12-2.
In the FDA Guidance for Industry on Pyrogen and Endotoxins Testing: Questions and Answers, Recombinant Horseshoe Crab Factor C is included as an alternative method in Section 5.
Guidelines for validation of alternative methods can be found in the United States Pharmacopoeia (USP) chapter <1225> and Ph. Eur. chapter 2.6.14.
General information on performing bacterial endotoxin testing can be found in the harmonized chapters of the USP <85>, Ph. Eur. 2.6.14 and Japanese Pharmacopoeias (JP) <4.01>.

Patent information

Parts of this product are licensed under the following patents: BR0210681, US6849426, AU2002330860, CN100390193, JP5039729.

11. Index of Symbols

Symbol	Meaning
	Catalog number
	Manufacturer
	Date of manufacture
	Temperature limit
	Use by date
	Batch code
	Consult Instructions for Use
	Contains sufficient for <n> tests
	Do not re-use

12. Limited Warranty

Hyglos GmbH – a bioMérieux company – warrants the performance of the product for its stated intended use provided that all procedures for usage, storage and handling, shelf life (when applicable), and precautions are strictly followed as detailed in the instructions for use (IFU).
Except as expressly set forth above, Hyglos GmbH – a bioMérieux company – hereby disclaims all warranties, including any implied warranties of merchantability and fitness for a particular purpose or use, and disclaims all liability, whether direct, indirect or consequential, for any use of the reagent, software, instrument and disposables (the “System”) other than as set forth in the IFU.

13. Revision History

Change type categories:

N/A	Not applicable (First publication)
Correction	Correction of documentation anomalies
Technical change	Addition, revision and/or removal of information related to the product
Administrative	Implementation of non-technical changes noticeable to the user

Note: *Minor typographical, grammar, and formatting changes are not included in the revision history*

Release date	Part Number	Change Type	Change Summary
2018/04	049458-01	N/A	Not applicable (First publication)

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