REF 890030 200254 V1.0 – en – 2017/02 EN

EndoZyme® II

Recombinant Factor C Endotoxin Detection Assay

Fluorescence microplate assay using Recombinant Horseshoe Crab Factor C (rFC).

Package Insert EndoZyme® II

Table of Contents

1. General Information

1.1 Intended Use

Intended use

EndoZyme® II is intended for quantitative determination of endotoxin (chemically lipopolysaccharide, LPS) in pharmaceutical end-products, in-process control and research samples, and medical device testing.

1.2 Test Principle

EndoZyme® II 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
Quantitation limit 0.005 EU/mL
Assay time 60 minutes

2. Kit Components

Number of tests

The kit contains reagents for 192 tests.

Kit components

Component	Container	Content	Description
1 Enzyme **(ENZ)**	Plastic bottle, transparent cap	1 x 2.5 mL	Enzyme (rFC) solution, 10-fold concentrated. 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, 10-fold concentrated.
3 Endotoxin Standard (**CSE**)	Glass bottle, orange cap	2 bottles	Endotoxin standard, lyophilized, containing approx. 100 EU of LPS from *Escherichia coli* O55:B5.
4 Water (**WEF**)	Plastic bottle, blue cap	2 x 100 mL	Water, free of detectable levels of endotoxin, for reconstitution of the standard and, dilution of standard and 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.
6 Assay Plate (**MPL**)	Plastic bag	2 plates	Sterile plates, free of detectable levels of endotoxin 2 x 96 wells.

3. Warnings and Precautions

Warning:
EndoZyme® II 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).

Endotoxin-free conditions
All materials used, such as containers or pipette tips, should be free of detectable levels of endotoxin. For preparing sample and standard dilutions, 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 aluminium caps or aluminium 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 test reagents must be free of detectable levels of endotoxin

4. Additional Reagents, Equipment, Instrumentation and Software Required

Equipment required
- Pipettes
- Multi channel pipette or dispensing pipette
- Pipette tips, endotoxin-free
- Glass test tubes, endotoxin-free (e.g. EndoGrade® Glass Test Tubes – Ref 800050)

Instruments

Vortex-type mixer
0-1500 rpm
To reconstitute the Endotoxin Standard (CSE), mix thoroughly by vortexing at 1400 rpm for 10 minutes. Sample dilutions and standard dilutions should be mixed vigorously for 2 minutes. 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 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	0.5 EU/mL: drfu ≈ 5% of maximum

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

Adjustment of instrument sensitivity (Gain)
When performing EndoZyme® II for the first time, the sensitivity setting (PMT gain) of the reader has to be adjusted specifically. **Do not use automatic gain adjustment.** The gain settings must be adjusted for each reader individually. The optimum standard curve is achieved when the signal increase of 0.5 EU/mL is adjusted to 5% of the maximum detectable signal of the reader.

Calculation software
For standard curve fitting and back-calculation of the endotoxin content of unknown samples, calculation software is required. Ideally, the EndoZyme® II standard curve is generated by fitting a linear regression function to the logarithmic data of both endotoxin concentration and net drfu (see section 6.7). Alternatively, a 4-parameter-logistic function can be used to extend the dynamic range to 50 EU/mL (see section 6.8).

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.

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 at +2-8°C
2 Substrate (**SUB**)	For Assay Reagent preparation	Stable until expiry date of the kit when stored at +2-8°C
3 Endotoxin Standard (**CSE**) (*E. coli* O55:B5)	The reconstitution volume is printed on the label; resolve lyophilized standard with water (4); mix for at least 10 minutes by vortexing	Stable for 4 weeks when stored at +2-8°C or until expiry date of the kit when stored frozen in aliquots at -20°C. Freeze and thaw only once.
4 Water (**WEF**)	Ready-to use	Stable until expiry date of the kit when stored at +2-8°C
5 Assay Buffer (**AB**)	For Assay Reagent preparation	Stable until expiry date of the kit when stored at +2-8°C

Reagents to be prepared from kit components

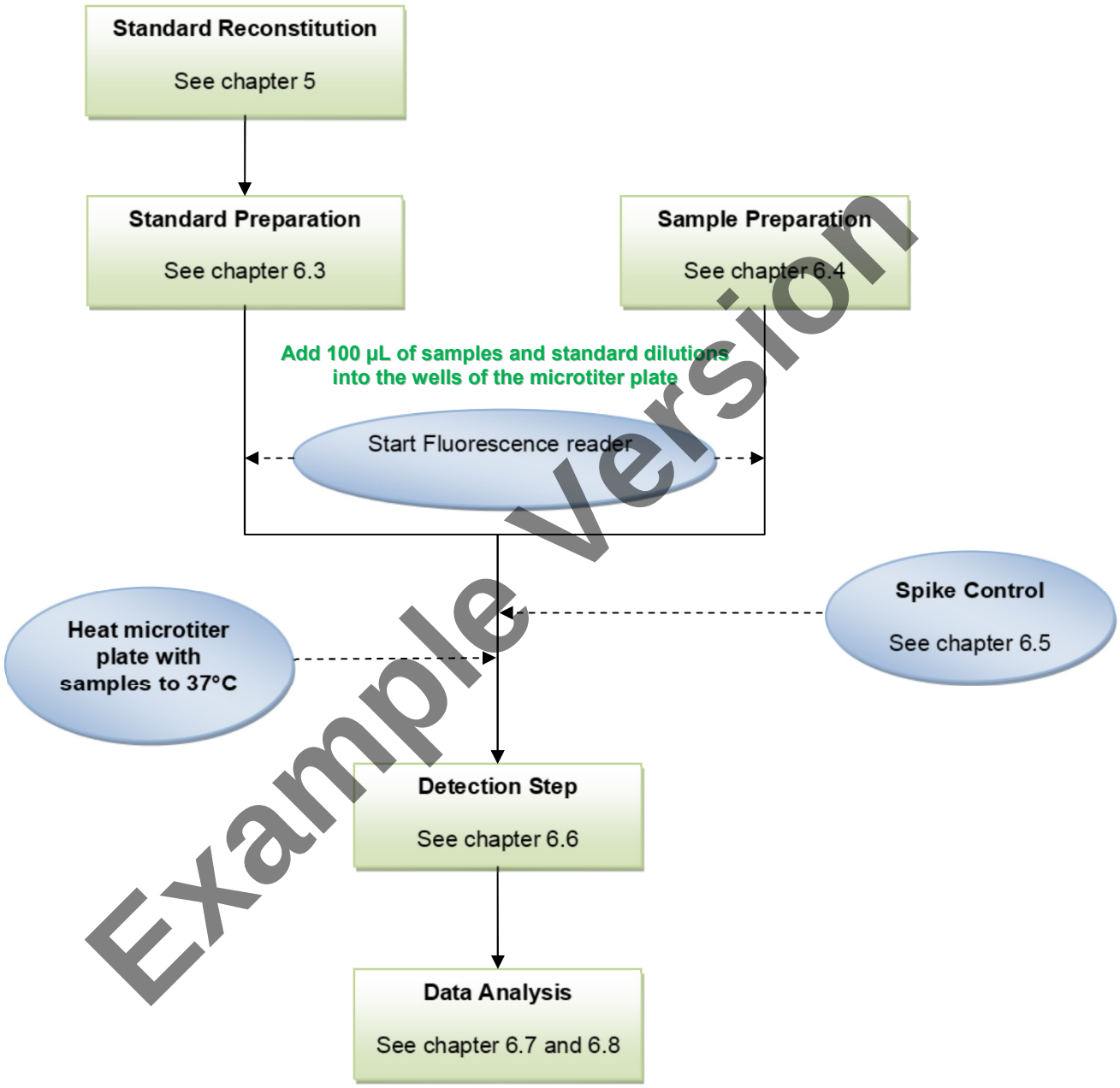
Reconstitution of Endotoxin Standard (CSE) :

- The volume to be used for reconstitution of the CSE (3) is indicated on the label.
- For reconstitution, pipette the indicated amount of Water (4) into bottle 3.
- **Important**: Use new pipette tips for every pipetting step to avoid contamination of the water.
- Close the bottle, mix thoroughly by vortexing at 1400 rpm for 10 minutes.

Assay Reagent: Prepare the Assay Reagent freshly immediately before use (see chapter 6.6 Assay Procedure for quantities).

6. Assay Protocol

6.1 Overview Assay Procedure



6.2 General Handling Instructions

Handling instructions

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

6.3 Standard Preparation

Serial dilution of Endotoxin Standard (CSE):

- The reconstituted CSE (3) has an activity of 50 EU/mL.
- For preparation of the dilution series, use endotoxin-free glass test tubes.

  Important: Dilution in plastic vials may lead to lower recovery at low endotoxin concentrations.

- Pipette 900 µL of water (4) into each tube prepared for the dilution series and the blank.
- Add 100 µL of the reconstituted CSE to prepare the second standard. Close the vial and mix thoroughly by vortexing at 1400 rpm for 2 minutes (resulting concentration is 5 EU/mL).
- Repeat the subsequent 1:10 dilution steps accordingly to prepare the remaining concentrations.
- Use water (4) as blank (negative control).
- Standard dilutions are stable for 8 hours when stored at 2-8°C.

Standard concentrations:

Depending on the used calculation method (see sections 6.7 and 6.8), different standard concentrations have to be prepared:

	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	+	+

6.4 Sample Preparation

Sample preparation/ sample dilution

Water samples can be analyzed undiluted. Other matrix compositions may interfere with the assay, i.e. inhibit or enhance the reaction (see section 6.10). To this end, interference testing is necessary. It is done by spiking diluted or undiluted sample with a known amount of endotoxin before the addition of assay reagent. The measured endotoxin concentration difference between spiked and unspiked sample (spike recovery, positive product control PPC) has to be between 50% and 200% of the amount of spiked endotoxin. Usually, sample matrix interference is dependent on concentration of matrix components other than LPS. In most cases, it can be overcome by sample dilution in endotoxin-free water.

Note: For highly complex sample matrices we recommend using the EndoLISA® Endotoxin Detection Assay.

- For sample dilution, use endotoxin-free 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. Vortex for at least 1 minute.

6.5 Spike Control

Spiking of samples Spiking of samples should be applied in order to validate, if sample components interfere with the assay and if dilution is required (see section 6.10 for interference parameter).

Spike material The Endotoxin Standard (CSE) provided with the kit can be used for spike control (PPC, Positive Product Control).

Spike concentration The Endotoxin concentration of the spike should equal or close to the centre of the standard curve. A spike concentration of 5 EU/mL or 0.5 EU/mL is common.

Recommended protocol

- Pipette at least four replicates of the sample, 100 µL each.
- Add 10 µL of the 50 EU/mL or the 5 EU/mL CSE to half of the samples wells (at least two).
- Proceed as described in section 6.6 assay procedure.

Validity criteria

A result is considered valid if the spike recovery is in the range of 50% to 200%. Samples with insufficient spike recovery need to be diluted.

6.6 Assay Procedure

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

Apply the indicated volumes in an endotoxin-free reagent reservoir:

Assay Reagent	Assay Buffer	Substrate	Enzyme
2 mL for 16 reactions	1.6 mL	0.2 mL	0.2 mL
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

Start reader

- Allow the fluorescence reader to warm up to 37°C

Filling of assay plate

- Select the required number of wells. Duplicate determinations are recommended.
- Pipette 100 µL of sample or standard-dilution into the respective wells.
- Perform spiking as described in 6.5.
- Warm up the microplate with samples to 37°C

Detection step

- Prepare 100 µL Assay Reagent for each well (described above).
- Place the microplate in or close to the reader and 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 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 plate at 37°C (incubator or fluorescence reader).
- Read fluorescence signals after 60 minutes (second reading).

Note: Longer reaction time may increase the sensitivity of the test

6.7 Standard Curve analysis using Linear Regression Model

Data export Export data (time point zero and time point 60 minutes) into a spreadsheet program.

Blank correction Subtract time point zero data from time point 60 minute data (drfu).
Calculate the mean drfu of the blank.
Subtract the mean blank drfu from the drfu of standards and samples (net drfu).
Calculate the logarithm of mean standard net drfu and concentrations (EU/mL).

Standard curve Plot the standard curve (log(EU/mL) vs. log(net drfu)).
Calculate curve function by fitting a linear model to the data:

$$\log(Y) = A*\log(X)+B$$

Calculate the correlation coefficient (r should be at least 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 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.

6.8 Standard Curve analysis using 4-Parameter Logistic Regression Model

Rationale To extend the measurement range by one order of magnitude, a standard at 50 EU/mL (concentration of reconstituted CSE) can be included. However, it is beyond the linear range of the assay. In this case, a non-linear regression model can 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:

$$Y = (A-D)/(1+(X/C)^B)+D$$

fit weight: 1/y

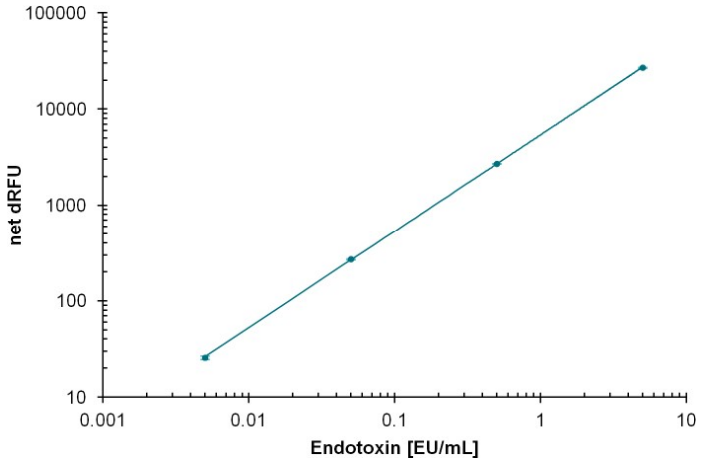
Calculate endotoxin concentration (EU/mL) of samples using the non-linear function.
Calculate the correlation coefficient (r should be at least 0.980).

Spike Recovery Calculate the endotoxin concentration difference between 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.

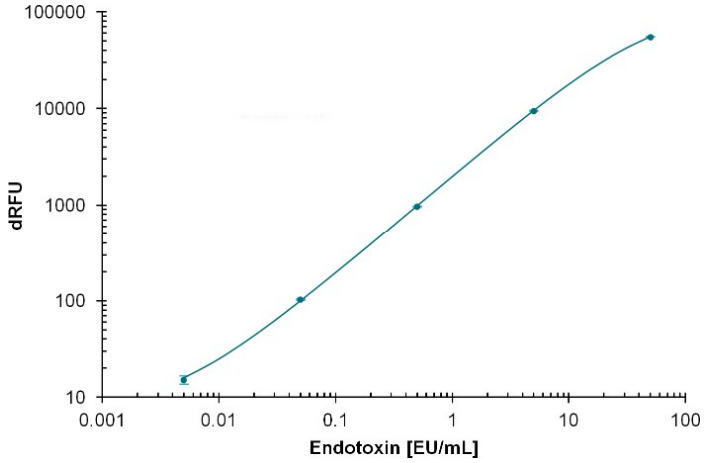
6.9 Standard Curve Examples

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

Linear regression



4-parameter logistic regression



6.10 Influencing Parameters and Limitations

Test interference can cause invalid results, as revealed by invalid spike recovery (see section 6.4) 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:

$$MVD = \frac{\text{Endotoxin limit}}{\text{Assay sensitivity}}$$

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 or 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 and be detected with the EndoLISA® assay, i.e. allow for 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 recommended.
Detergents	Detergents may interfere with EndoZyme® II. 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. By absorbing endotoxin, proteins may stimulate LER.

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, 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 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 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.hyglos.com).

9. Trouble Shooting 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 standard or sample pipetted)	Repeat assay
	Interfering ingredients	Spike control; dilute sample 1:10
	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

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 Pharmacopeia chapter <1225> and Ph.Eur. chapter 2.6.14.
General information on performing bacterial endotoxin testing can be found in the harmonized chapters USP <85>, Ph.Eur. 2.6.14 and Japanese Pharmacopoeia (JP) <4.01>.

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

11. Index of Symbols and Abbreviation

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

Abbreviations used:

CSE	Control Standard Endotoxin (Endotoxin Standard supplied with kit)
drfu	Difference in relative fluorescence units between time points of a single measurement
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EU	Endotoxin Unit (1 EU corresponds to 0.1 ng LPS (FDA RSE *E. coli* O113 EC-6)
FDA	Food and Drug Administration
JP	Japanese Pharmacopoeia
LAL	*Limulus* amebocyte lysate
LER	Low Endotoxin Recovery
LPS	Lipopolysaccharide
Ph.Eur.	European Pharmacopoeia
rFC	Reconbinant Factor C
rfu	Relative fluorescence unit
rpm	Revolutions per minute
RSE	Reference Standard Endotoxin
TAL	*Tachypleus* amebocyte lysate
USP	United States Pharmacopeia

12. Limited Warranty

Hyglos / bioMérieux 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 / bioMérieux 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
	Minor typographical, grammar, and formatting changes are not included in the revision history.

Release date	Part Number	Change Type	Change Summary
2017/02	200254 V1.0	N/A	Not applicable (First publication)

Hyglos GmbH
- a bioMérieux company
Am Neuland 3
D-82347 Bernried am Starnberger See
Germany

HRB 178396, München
Tel: +49 (0)8158 9060 0
Fax: +49 (0)8158 9060 210
www.biomerieux.com