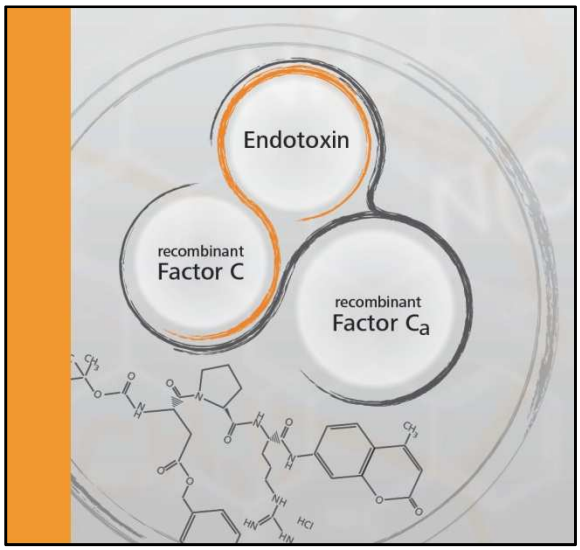
Package Insert

Haemotox® rFC

Article No.: 31310

Storage: +2-8°C

For laboratory and research use only
Not for use in diagnostic procedures



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Abbreviations

dRFU	Difference in relative fluorescence units between time points of a single measurement
net dRFU	Difference in relative fluorescence units between time points of a single measurement (Blank corrected)
EDTA	Ethylen diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EU	Endotoxin Unit (1 EU ≈ 0.1 ng LPS (FDA RSE *E. coli* O113 EC-6))
LAL	Limulus amebocyte lysate
LPS	Lipopolysaccharide
MTP	Microtiter Plate
RFU	Relative fluorescence unit
rpm	Revolutions per minute
RSE	Reference Standard Endotoxin

1 General Information

1.1 Intended Use

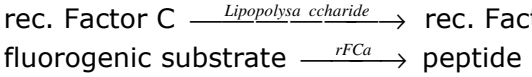
Haemotox® rFC is intended for *in vitro* quantitative determination of lipopolysaccharide (LPS) in pharmaceutical and biological end products, in-process control and research samples as well as for medical device testing.

Haemotox rFC® is a homogeneous enzymatic assay which uses the LPS receptor (recombinant Factor C) of the blood clotting cascade of horseshoe crab in combination with a fluorogenic substrate.

Warning: For laboratory and research use only. Haemotox® rFC is not intended for use with clinical samples or for diagnosis of human or animal disease.

1.2 Test Principle

Endotoxin	Endotoxins are bacterial cell membrane constituents which are recognized by the human immune system and 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, 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. Factor C is a zymogene (precursor of a protease) that is activated by endotoxin.
Factor C	Recombinant Factor C (rFC) is used instead of amebocyte lysate in combination with a synthetic fluorescence substrate for detection of LPS.



1.3 Specifications

Assay range	0 to 50 EU/mL
Detection limit	0.005 EU/mL, as defined by the lowest standard concentration
Assay time	60 minutes
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 in chapter 5.
Validation	Haemotox® rFC performance parameters were confirmed by validation studies. Validation data are available on request.

1.4 Kit Content

Components	Container	Content	Description
1 Enzyme	Plastic bottle, transparent cap	1 x 2,5 mL	Enzyme solution for the detection of LPS, 10-fold concentrated
2 Substrate	Brown plastic bottle, brown cap	1 x 2,5 mL	Fluorescence substrate, 10-fold concentrated
3 Endotoxin Standard	Glass bottle, orange cap	2 bottles	Endotoxin Standard, lyophilized, containing approx. 100 EU LPS from *E. coli* O55:B5
4 Water (Endotoxin-free)	Plastic bottle, blue cap	2 x 100 mL	Endotoxin-free water for reconstitution of the standard and for dilution of standard or samples
5 Assay Buffer	Brown plastic bottle, brown cap	2 x 12 mL	Assay buffer, to be combined with Substrate (2) and Enzyme (1)
6 Microtiter-plate	Plastic bag	2 plates	Sterile and Endotoxin-free plates, 2 x 96 wells

2 Safety information

All of the kit components are not considered to be hazardous, but have not been tested considering their toxicological properties. A Material Safety Data Sheet (MSDS) is available on request.

3 Avoiding Contaminations

Endotoxin-free Conditions	All materials used, such as containers or pipette tips, should be purchased endotoxin-free. 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 or 250°C for 1 h. 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 contamination. All materials in direct contact with the sample or test reagents must be endotoxin-free.

4 Instrumentation and Software

Vortex

Standard dilutions and sample dilutions have to be mixed vigorously. This is optimally achieved by using a multi-tube vortexer (e.g. Heidolph Multi Reax test tube shaker).

Incubator

The incubation of the assay plate should ideally be performed in the MTP 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 reader from different suppliers may be used for reading of Haemotox® rFC results. Haemotox® rFC has been developed and validated on an FLx800™ fluorescence microplate reader from BioTek Instruments. All validation results have been generated on this instrument with settings as described below. Using other hardware, these instrument parameters may be used as orientation but should be validated.

Instrument settings:

Temperature	37°C
Excitation Filter(nm/band)	380/20
Emission Filter (nm/band)	440/40
Optics position	top
Readings per well	min. 10
Shaking mode	On
(15 seconds medium shaking before reading time point zero)	
Sensitivity/PMT gain	0.5 EU/mL: dRFU ≈ 5% of max.

Note: Suggested gain setting applicable for standard curve from 0.005 – 5.0 EU/mL

Adjustment of instrument sensitivity (Gain)

When performing Haemotox® rFC for the first time, the sensitivity setting (gain) of the fluorescence reader has to be adjusted. 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. For example, the range of the BioTek Instruments FLx800™ reader is between 0 and 99.999 RFU. For this instrument, 0.5 EU/mL should be adjusted to dRFU = 5.000 RFU.

Attention: The gain settings must be adjusted for each reader individually and should be checked for each different kit lot.

Calculation Software

For standard curve fitting and back-calculation of the endotoxin content of unknown samples, a calculation software is required. Ideally the Haemotox® rFC standard curve is generated by fitting a linear regression function to the logarithmic data of both endotoxin concentration and net dRFU. Alternatively, a 4-parameter-logistic function can be used to extend the dynamic range to 50 EU/mL.

When using readers from BioTek Instruments, Gen5™ software is the preferential means of calculating the standard curve. The software settings can be requested from Haemochrom Diagnostica.

5 Reagent Preparation

5.1 Use of Kit Components, Storage and Stability Conditions

Reagent	Preparation	Stability and storage conditions of working solutions
1 Enzyme	For Assay Reagent preparation	Stable until expiry date of the kit when stored at +2-8°C
2 Substrate	For Assay Reagent preparation	Stable until expiry date of the kit when stored at +2-8°C
3 Endotoxin Standard	• Reconstitution volume see label • Resolve lyophilized standard with printed quantity of endotoxin-free water (bottle 4) • Vortex for at least 10 minutes	• Stable for 4 weeks when stored at +2-8°C • in Aliquots at -20°C until expiry date of the kit • Freeze and thaw only once
4 Water (Endotoxin-free)	Ready to use	Stable until expiry date of the kit when stored at +2-8°C
5 Assay Buffer	For Assay Reagent preparation	Stable until expiry date of the kit when stored at +2-8°C

5.2 Reagent Preparation

5.2.1 Reconstitution of LPS Standard

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

5.2.2 Assay Reagent

Prepare the Assay Reagent freshly immediately before use. Required amounts are indicated in the table below. Combine 8 parts of Assay Buffer (bottle 5), 1 part of Enzyme (bottle 1) and 1 part of Substrate (bottle 2). (Necessary amount of reagent dependent on the number of wells used, see table below.)
Mix carefully - **do not vortex**.

Number of reactions	Necessary Assay Reagent [mL]	Assay Buffer [mL]	Substrate [mL]	Enzyme [mL]
16	2	1.6	0.2	0.2
32	4	3.2	0.4	0.4
48	6	4.8	0.6	0.6
64	8	6.4	0.8	0.8
80	10	8.0	1.0	1.0
96	12	9.6	1.2	1.2

6 Assay Protocol

6.1 Handling Instructions

General Instructions
- All reagents needed for running Haemotox® rFC 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 in duplicates.
- Reagents from different lots **must not** be mixed and used in one test series.

Equipment required
- Pipettes
- Multi channel pipette or dispensing pipette
- Pipette tips, endotoxin-free
- Glass test tubes, endotoxin-free (e.g. Haemotox® Sample Tubes)

Instruments
- Vortexer, 0-1500 rpm
- Fluorescence microplate reader, temperature controlled
- Incubator 37°C (optional, needed when microplate reader lacks temperature control)

6.2 Standard Preparation

Serial Dilution of LPS Standard
- The reconstituted LPS Standard has a concentration of 50 EU/mL.
- For preparation of the dilution series, use endotoxin-free glass test tubes. **Important:** Dilution in plastic vials may lead to reduced recovery at low concentrations.
- Pipette 900 µl of endotoxin-free water into each tube prepared for the dilution series and the zero standard (blank).
- Add 100 µl of the reconstituted LPS Standard to prepare the second standard. Close the vial and mix thoroughly by vortexing at 1400 rpm for 1 minute (resulting concentration is 5 EU/mL).
- Repeat the subsequent 1:10 dilution steps accordingly to prepare the remaining concentrations.
- Use endotoxin free water as zero standard (blank).
- Standard dilutions are stable for 8 h when stored at +2-8°C.

Standard concentrations
Depending on the standard concentrations prepared different calculation methods have to be used:

Standard [EU/mL]	Non-linear regression	Linear regression
50	+	-
5	+	+
0.5	+	+
0.05	+	+
0.005	+	+
0	+	+

6.3 Sample Preparation

Sample preparation/sample dilution
Water samples can be analyzed undiluted. Other matrix compositions may interfere with the assay (chapter 6.9). To this end, interference testing is necessary. Interference (inhibition/enhancement) testing of endpoint fluorescent methods is done by spiking a sample or diluted sample with a known concentration of endotoxin and testing for spike recovery in duplicate by the supplier's instructions (chapter 6.4). Usually, sample matrix interference is dependent on concentration of matrix components other than LPS. In most cases, inhibition/enhancement can be overcome by sample dilution in endotoxin-free water. For complex sample matrices an additional sample pre-treatment may be required to overcome interference. For sample dilution, use endotoxin-free glass test tubes.

For example, a 1:10 dilution should be prepared as follows:
Pipette 900 µl of endotoxin-free Water (bottle 4) into a vial and add 100 µl of sample. Vortex.

6.4 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 (chapter 6.9 for interference parameter).
Spike material	The LPS Standard provided with the kit has to be used for spike control.
Spike concentration	Select an endotoxin concentration at or near the middle of the endotoxin standard curve, depending on the dilution of the sample and its expected endotoxin content.
Recommended Protocol	• Add four replicates of the sample to the plate, 100 µl each. • Add 10 µl of the 5 EU/mL or the 50 EU/mL standard to two of the four wells (equals 0.5 EU/mL or 5 EU/mL in the respective well). • Proceed as described in chapter 6.5 assay procedure.
Validity criteria	A result can be considered as valid, if the spike recovery is in the range of 50% to 200%. Samples with invalid spike recovery have to be diluted or treated differently before testing.

6.5 Assay Procedure

Start Reader	Allow reader to warm up for min. 20 minutes.
Filling of assay plate	• Select the required number of wells. At least, duplicate determinations are recommended. • Pipette 100 µl of blank, sample or standard dilution into the respective wells. • Perform spiking as described in chapter 6.4. • Warm up the filled microplate to 37°C.
Detection	• Prepare more than 100 µl Assay Reagent for each well (see 5.2.2). • Add 100 µl of Assay Reagent to each well while the microplate is placed in the reader. • Close the reader and wait 1 min to allow the temperature to adjust. • 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). **Recommended:** Use a dispensing pipette or a multi-channel pipette in order to reduce hands-on time. **Note:** Longer reaction time may increase the sensitivity of the test.

Overview Assay Procedure

Start Fluorescence Reader to warm up to 37°C		
Standard Reconstitution and Dilution Chapter 5.2.1 and 6.2	Sample Preparation Chapter 6.3	Spike Control Preparation Chapter 6.4
Add 100 µl of prepared standards, samples and controls into the wells of the microtiter plate		
Incubate plate with samples to warm to 37°C		
Preparation of Assay Reagent Chapter 5.2.2		
Add 100 µl of Assay Reagent to each well		
Detection		
Calculation		

6.6 Standard Curve Analysis using Linear Regression Model

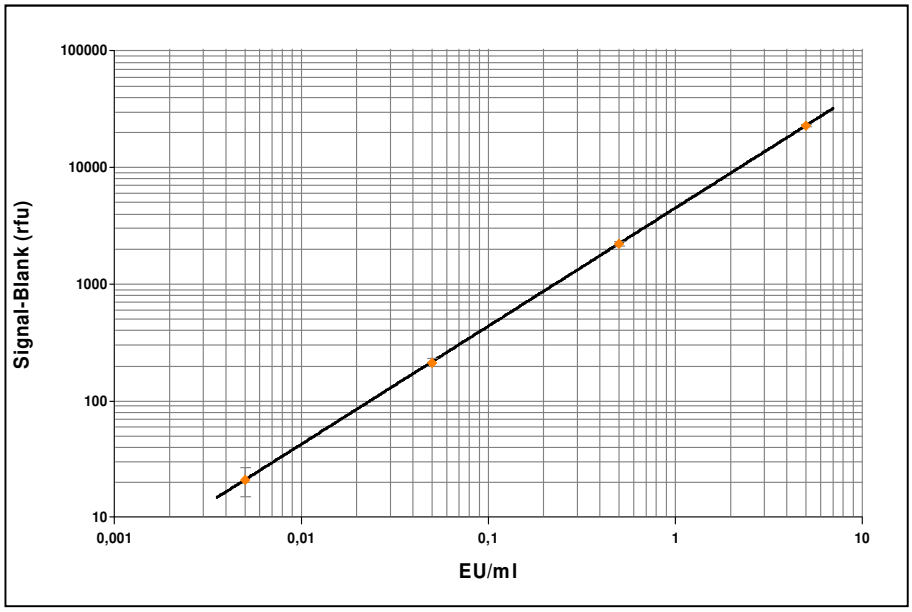
Blank correction	• Subtract time point zero data from time point 60 minute data. • Calculate the mean dRFU of the blank. • Subtract the mean blank dRFU from the dRFU of standards and samples (net dRFU). • Calculate the logarithm of the mean standard net dRFU and concentrations [EU/mL].
Standard curve	• Plot the standard curve (log [EU/mL] vs. log [net dRFU]). • Calculate function by fitting to a linear equation: $\log(Y) = A*\log(X) + B$ • Calculate regression coefficient ($R^2 \geq 0.980$).
Sample values	• Calculate EU/mL values of samples on the basis of the linear equation. • Multiply results with the dilution factor 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.

6.7 Standard Curve Analysis using 4-Parameter Logistic Regression Model

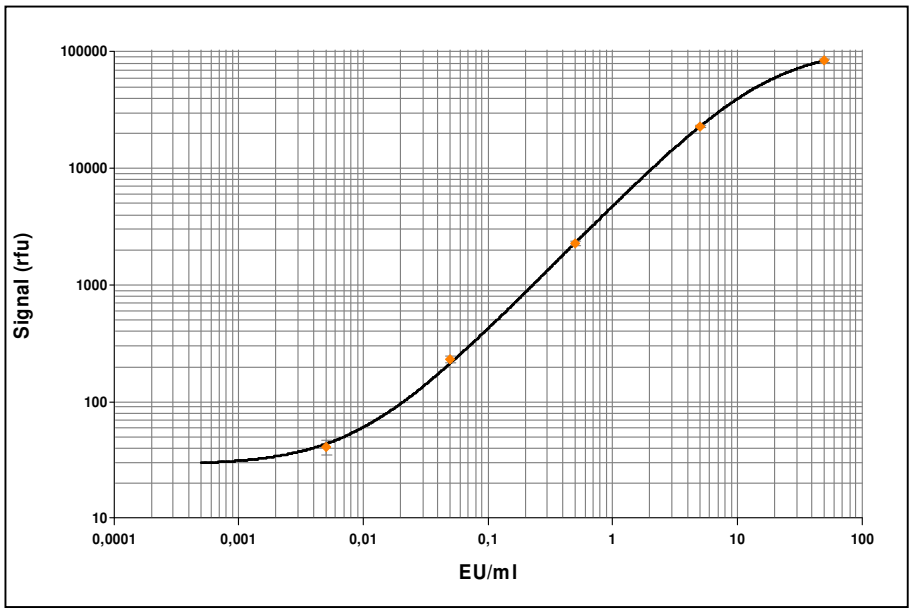
Rationale	• To extend the measurement range by one order of magnitude, a standard at 50 EU/mL (concentration of reconstituted standard) can be included. However, it is beyond the linear range of the assay. In this case, a non-linear regression model can be fitted to the data.
Data handling	• Subtract 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 the endotoxin concentration [EU/mL] of samples using the non-linear function. • Calculate the correlation coefficient ($R^2 \geq 0.980$).
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.

6.8 Typical Standard Curves

1. Haemotox® rFC Standard curve:
Linear regression, generated by Gen5™ Software)



2. Haemotox® rFC Standard curve:
4-Parameter logistic non-linear regression, generated by Gen5™ Software



6.9 Influencing Parameters and Limitations

Test interference can cause invalid results, as revealed by invalid spike recovery. The two mayor causes for interference are alteration of the enzyme reaction conditions and alteration of the LPS aggregation state of the spike control. Normally, such interference can be overcome by sample dilution in endotoxin-free water. If interference cannot be overcome by diluting the sample within the maximum valid dilution (MVD) of a given product additional pre-treatment of the product is necessary to overcome the test disturbances.

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

Temperature	For the detection reaction the temperature of 37°C is mandatory. 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 value	Samples having extreme pH values may influence assay performance if the buffer capacity of the test system is exhausted. Interference can be investigated by spiking experiments. In the case of invalid spike recovery, dilution or pH adjustment to pH 6-8 is required.
Salt concentration	Total salt concentration in a sample should not exceed 500 mM. In case of higher concentration dilution is required.
Detergents	Detergents may interfere with the Haemotox® rFC test. Interference can be checked by spiking experiments. In case of invalid spike recovery additional dilution of the sample or other pre-treatment is required.
Chelating agents	Chelating agents (e.g. EDTA, EGTA, and citrate) in the sample will interfere with the assay. If such agents are present, dilution is required or the chelating agent has to be neutralized with e.g. magnesium. Interference can be checked by spiking experiments.
Chaotropic agents	Chaotropic agents may interfere with the Haemotox® rFC test and denature Factor C. Interference can be checked by spiking experiments. In case of invalid spike recovery further dilution or other pre-treatment is required.
Organic solvents	Interference of organic solvents has to be tested. Interference can be checked by spiking experiments. In case of invalid spike recovery further dilution or other pre-treatment is required.
Proteins	Proteins at high concentrations may interfere with the assay. Protein interference strongly depends on the physical and chemical properties of the proteins. Interference by proteins should be checked using spiking controls.
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 Haemotox® rFC, 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.
Biological samples	Haemotox® rFC is not suitable for the detection of endotoxin in serum, plasma or blood samples.

7 Trouble Shooting Guide

Observation	Possible Cause	Measure
No signal at all	• Wrong instrument settings • Lamp defect • Pipetting error • Incubation temperature too high or much too low	⇨ Check instrument parameter ⇨ Change lamp ⇨ Check reagents, repeat assay ⇨ Check temperature settings
No signal with individual samples	• Pipetting error (no standard or sample pipetted) • Interfering ingredients • Inappropriate pH value	⇨ Repeat assay ⇨ Spike control, dilute sample 1:10 ⇨ Check pH value, neutralize sample
Low signal level	• Wrong sensitivity adjustment (gain) • Reader defect, e.g. optics • Incubation temperature too high/too low • Kit damage (shipment or storage) • Kit or working solutions expired • Inappropriate emission wavelength or band	⇨ Adjust sensitivity ⇨ Run instrument check ⇨ Check temperature ⇨ Increase pre-incubation of the samples in the plate at 37°C to 10 minutes ⇨ Check storage conditions and package material, contact technical service ⇨ Use new kit or fresh reagents ⇨ Emission filter should be at 440 nm, band should be 40 nm
High background signal in standards and negative controls	• LPS contamination of assay components e.g. water • LPS contamination of vials or pipette tips • Inappropriate excitation wavelength or band	⇨ Use fresh reagents ⇨ Use different lot of vials and pipette tips, switch to glass vials or change supplier ⇨ Excitation filter should ideally be at 380 nm, band should be 20 nm
High well to well variation	• Temperature gradient (incubator, reader) • Pipette damage	⇨ Change incubator/reader ⇨ Calibrate pipettes
Invalid spike control	• Interfering ingredients • Inappropriate pH value	⇨ Dilute sample ⇨ Check pH, neutralize sample

8 Endotoxin-free related products

- **Haemotox® Sample Tubes**
  Borosilicate-glass test tubes applied to dilution of endotoxin standards as well as to dilution and aliquotation of samples.

LPS content:	< 0.001 EU/mL.
Height	100 mm
Outer diameter	13 mm
Amount	50
Article No.:	**31331**

- **Haemotox® Sample Tubes**
  Borosilicate-glass test tubes with metal cap applied to dilution of endotoxin standards as well as to dilution and aliquotation of samples.

LPS content:	< 0.001 EU/mL.
Height	100 mm
Outer diameter	16 mm
Amount	10
Article No.:	**31333**

- **Haemotox® Sample Tubes with metal cap**
  Borosilicate-glass test tubes with metal cap applied to dilution of endotoxin standards as well as to dilution and aliquotation of samples.

LPS content:	< 0.001 EU/mL.
Height	90 mm
Outer diameter	16 mm
Amount	70
Article No.:	**31336**

- **Haemotox® Water**
  Sterile, ultra-pure water. Recommended for endotoxin-free reconstitution and dilutions of reagents, samples and standards used in endotoxin analytics.
  LPS content: < 0.001 EU/mL.

Haemotox® Water 30 mL	20 x 30 mL
Article No.:	**31222**
Haemotox® Water 100 mL	16 x 100 mL
Article No.:	**31223**
Haemotox® Water 500 mL	6 x 500 mL
Article No.:	**31224**

- **Endotoxin-free Containers**
  Certified endotoxin-free containers for sample storage

  30 mL, unlabeled, ca. 400 containers
  Article No.: **AB128**

9 Distribution and Technical Service

For order, more information or technical assistance contact to:

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Guidelines for the test of bacterial endotoxins can be found in the European Pharmacopoeia, chapter 2.6.14 in combination with chapter 5.1.10 and in the US Pharmacopoeia, chapter <85>.

10 Regulatory Information

Trademarks

Haemotox® is a registered trademark of Haemochrom Diagnostica GmbH.
FLx™ and Gen5™ are trademarks of BioTek Instruments.

11 Revision Follow-Up

Date	Chapter	Change
Revision 01_09 2019		
Sept. 2019	4	Vortex: Time specification removed
Sept. 2019	4	Adjustment of instrument sensitivity (Gain): Check gain settings after Kit batch change
Sept. 2019	6.3	Vortex of sample dilutions: Time specification removed
Sept. 2019	8	Update of available consumables

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