ROCKLAND

EndoAlert™ Endotoxin ES Kit Protocol

KMA-0200 Endotoxin ES

The EndoAlert™ ES Plate Kit is a kinetic, colorimetric assay for the quantitative determination of bacterial Endotoxin in aqueous solutions. Endotoxin, a bacterial lipopolysaccharide, is one of the major cell wall components of most gram-negative bacteria. The EndoAlert Plate Kit detects low levels of Endotoxin and is therefore a useful tool to assess the integrity of biological and environmental samples. The detection ranges from 0.01–10 Endotoxin units (EU/mL).

The EndoAlert Plate Kit is a quantitative version of the reaction first described by Levin and Bang in 1968. The test is based upon an enzymatic cascade where Endotoxin activates Factor C in Limulus Amebocyte Lysate (LAL) which in turn activates Factor B. Factor B activates Proclotting Enzyme which then activates Clotting Enzyme. A colorless synthetic peptide substrate is hydrolyzed by Clotting Enzyme to generate a yellow color which can be measured by a spectrophotometer at 405 nm. The degree of color resulting from the reaction is proportional to the amount of Endotoxin in the test sample and can be calculated using a standard curve.

I. Reagents & Equipment Provided

Product	Preparation	KMA-0200
96-well Microtiter Plate	N/A	1
Endotoxin Standard	Lyophilized 10EU/vial	1
Chromogenic Lysate	Lyophilized	3
LAL Reagent Water (LRW)	10 mL	1
Endotoxin Specific Reconstitution Buffer	3.5 mL	1

II. Reagents & Equipment Required but Not Provided

Note: All disposable materials must be free of interfering Endotoxin. Any glassware used must be depyrogenated under dry heat (250°C for at least 0.5 hours is recommended).

Product
Pipette with disposable tips capable of dispensing 50 µL
Pipette with disposable tips capable of dispensing 100-1000 µL
Repeater pipette with disposable tips capable of dispensing 50 µL (optional)
Pyrogen free glass test tubes (12 X 75 mm) for dilutions
Parafilm®
Vortex Mixer
Timer
Microplate reader capable of reading kinetically at 405 nm and incubates at 37°C (ex. Molecular Devices SpectraMax Plus 384).

III. Storage

Store all kit reagents at 2-8°C in the dark. Avoid storing kits for extended periods (i.e. over 24 hours) at room temperature. Reagents should be brought to room temperature, 20–28°C (62 to 82°F) prior to use. Reconstituted Chromogenic Lysate should be stored at 2-8°C and used within 24 hours. Alternatively, reconstituted Chromogenic Lysate can be frozen at -20°C for up to 30 days, thawed once and used immediately. Diluted standards should be used within 8 hours.

IV. Kit Handling Notes & Precautions

- Perform all steps of the assay procedure using aseptic technique in a laminar flow hood.
- Running diluted standards, samples and controls in duplicate will improve assay precision and accuracy.
- Precise transfer of samples and reagents by using an appropriate and calibrated pipette requires constant monitoring of technique and is critical to obtain proper assay results.

- The use of a repeater pipette to dispense the Chromogenic Lysate when running 8 or more wells is highly recommended.
- Vortex each solution prior to pipetting to ensure accurate measurement of endotoxin concentration.
- Always keep the lid on the 96-Well Microtiter Plate except when adding reagents to avoid accidental contamination.
- Be sure to remove the 96-Well Microtiter Plate lid prior to placing the plate in the reader to avoid optical interference from condensation.
- Dilution or adulteration of reagents or samples not called for in the procedure may result in inaccurate results.
- Each reagent is optimized for use in the EndoAlert Plate Kit. Do not substitute reagents from any other manufacturer into the test kit. Do not combine reagents from other EndoAlert Plate Kits with different lot numbers.
- Avoid using any reagents beyond their expiration date.
- Do not use any kit components that have been damaged or contaminated.
- Wear suitable protective clothing and gloves.
- Establish a clean environment for assay manipulation. All materials and reagents should be free of interfering levels of Endotoxin to eliminate cross contamination. Note that glucan and fungal contamination from the human body, clothes, containers, water, and airborne dust may also cause interference with the EndoAlert Plate Kit.
- The EndoAlert Plate Kit provides a level of Endotoxin only in relation to the standard. It is NOT specific to the species of gram-negative bacteria which is the source of the Endotoxin in the sample. To increase accuracy of the test when the source of Endotoxin is known, use a purified Endotoxin from that species.
- The presence of high concentrations of (1,3) β-D-Glucan may elicit a false positive reaction.

V. Sample Preparation

Care should be taken to avoid contamination of the sample with interfering levels of Endotoxin. Be sure to use aseptic technique and only use pyrogen free collection materials. If samples are not able to be tested promptly following collection, store samples at 2-8°C for less than 24 hours or ≤ -18°C for periods of 24 hours or longer. Samples should be stored in appropriate collection containers to ensure accurate measurement of Endotoxin concentration as Endotoxin may adsorb to the inner surface. The pH of the sample should be within the range of 6.0–8.0. Sample pH may be adjusted using pyrogen-free sodium hydroxide or hydrochloric acid. Water and simple salt solutions do not require pH adjustments. To avoid contamination, test the pH of a small aliquot of the bulk sample solution.

VI. Reagent Preparation

Perform all steps of the reagent preparation using aseptic technique in a laminar flow hood. Reagents should be brought to room temperature, 20–28°C (62–82°F) prior to use.

Endotoxin Standards:

The EndoAlert Plate Kit is optimized for linearity between 0.01 EU/mL and 10 EU/mL. An example of standard dilutions can be found below. Alternative standard dilutions may be used to suit the users' requirements. A minimum of 3 standards is recommended.

1. Prepare Endotoxin standard curve in LRW at 10, 1, 0.1 and 0.01 EU/mL

Vial	Endotoxin Standard Concentration (EU/mL)	LRW (µL)	Endotoxin Standard Solution
A	10	1000	
B	1	450	50 µL of the 10 EU/mL standard
C	0.1	450	50 µL of the 1 EU/mL standard
D	0.01	450	50 µL of the 0.1 EU/mL standard
-	Negative Control	450	

2. Reconstitute the lyophilized Endotoxin Standard by adding 1 mL of LRW. Vortex vigorously for ≥ 20 seconds. Let sit for 15-20 minutes with occasional vortexing.
3. Prepare a 1 EU/mL endotoxin standard by adding 50 µL of the 10 EU/mL endotoxin standard to 450 µL LRW in a pyrogen free glass tube. Vortex vigorously for ≥ 20 seconds before proceeding.
4. Prepare a 0.1 EU/mL endotoxin standard by adding 50 µL of the 1 EU/mL endotoxin standard to 450 µL LRW in a pyrogen free glass tube. Vortex vigorously for ≥ 20 seconds before proceeding.
5. Prepare a 0.01 EU/mL endotoxin standard by adding 50 µL of the 0.1 EU/mL endotoxin standard to 450 µL LRW in a pyrogen free glass tube. Vortex vigorously for ≥ 20 seconds before proceeding.

Chromogenic Lysate:

1. Reconstitute each required vial of the Chromogenic Lysate by adding 1.1 mL of Endotoxin Specific Reconstitution Buffer.
2. Cover the vial with Parafilm ® and gently swirl to mix.
3. Let sit for 15-20 minutes.

Note: Confirm the Chromogenic Lysate is completely dissolved before proceeding. After reconstitution, the required vials may be combined to ensure consistency.

VII. Procedure

Note: Perform all steps of the assay procedure using aseptic technique in a laminar flow hood. Reagents should be brought to room temperature, 20–28°C (62–82°F) prior to use.

1. Prepare the plate reader using the following run parameters:

Wavelength	Temperature	Maximum OD	Onset OD	Run Time	Read Intervals	Shake
405 nm	37°C	0.5	0.03	60 minutes	30 seconds	Once before the first reading

2. Pipette 50 µL of each diluted standard, sample, and control into the appropriate wells. Running diluted standards, samples, and controls in duplicate will improve assay precision and accuracy.
3. Add 50 µL of reconstituted Chromogenic Lysate to each well and gently shake the plate by hand to mix. The use of a repeater pipette to dispense the Chromogenic Lysate when running 8 or more wells is highly recommended.
4. Place the plate into the 37°C plate reader with the lid off and begin kinetic assay data collection.
5. Collect and analyze the data for valid assay criteria.

VIII. Assay Criteria

- The correlation coefficient (R) of the standard (log vs. log) should be ≥ 0.980.
- If the RSD% for replicates are greater than 15%, the samples should be retested.
- If unknown samples are found to be out of range of the highest standard, perform additional dilutions so that they fall within range of the standard curve.

IX. Example Calculations & Standard Curve

Endotoxin Standard (EU/mL)	Time to Onset OD (seconds)	Average Reaction Time ± SD	RSD%	Back Calculation Endotoxin Concentration (EU/mL)
0.01	1628	1702 ± 52.0	3.1	0.010
	1741			
	1734			
	1708			
0.10	1036	1045 ± 12.0	1.1	0.097
	1051			
	1059			
	1034			
1.00	616	624 ± 8.4	1.3	1.070
	633			
	629			
	617			
10.00	389	389 ± 5.9	1.5	9.664
	394			
	390			
	381			

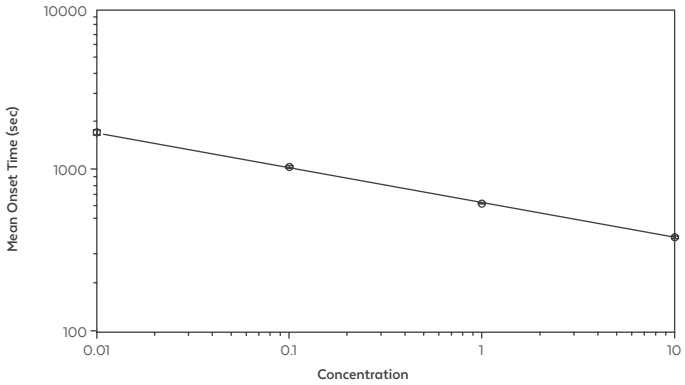

Figure 1. Sample Standard Curve EndoAlert ES Endotoxin Plate Kit: Mean Time (sec) vs. Concentration (EU/mL)

Log-Log fit: Log(y) = A + B * Log(x)

A	B	R^2
2.8	-0.215	1

Data obtained with Molecular Devices SpectraMax Plus and SoftMax Pro 4.5.6. Software Actual values may vary. This data is for example purposes only.
RSD% = (SD / Average Reaction Time) * 100