Endotoxin End-point Quantitation Kit

(End-point Chromogenic Assay)

Catalog Numbers: ECT032 (32 test)

ECT064 (64 tests)

PRODUCT DESCRIPTION

End-Point Endotoxin Quantitation assays are widely used for the *in vitro* detection and quantification of endotoxins, specifically lipopolysaccharides (LPS), which are the primary components of the outer membrane of Gram-negative bacteria. Bacteria release endotoxins in small amounts during growth and in larger amounts upon cell death. These highly pyrogenic molecules pose significant health and safety risks even at very low concentrations, as they can trigger strong inflammatory and immune responses, potentially leading to endotoxin shock in mammalian systems. Endotoxins are highly heat-resistant and can only be neutralized through extreme treatment measures. Contamination can stem from water, raw materials, laboratory equipment, containers, or non-aseptic procedures, posing serious risks to product safety and quality. Sensitive and reliable detection and control of endotoxins are essential in the manufacture of pharmaceuticals, biologics, and laboratory reagents.

The R&D Systems™ End-Point Endotoxin Quantitation Assay provides a highly sensitive and reliable *in vitro* method for accurately detecting and quantifying endotoxin levels in biological samples. The kit contains a Lyophilized Amebocyte Lysate (AL) Reagent, derived from circulating amebocytes of the Asian horseshoe crab *Tachypleus tridentatus*. When endotoxins are present in a sample, they activate proenzymes in the lysate, leading to the formation of coagulase enzymes. These enzymes catalyze the hydrolysis of a colorless substrate, resulting in the release of the yellow chromophore p-nitroaniline (pNA). The resulting color intensity is measured quantitatively at 405 nm, showing a linear correlation between absorbance and endotoxin concentration within the ranges of 0.01-0.1 EU/mL and 0.1-1.0 EU/mL.

INTENDED USE

For Research Use Only. Not for use in diagnostic procedures.

The Endotoxin End-Point Chromogenic Assay provides procedures for two endotoxin concentration ranges, 0.1-1.0 EU/mL and 0.01–0.1 EU/mL, determined by different incubation times. The AL Reagent is not labeled with a specific sensitivity; sensitivity is defined as the lowest endotoxin concentration used to construct the standard curve. The lowest detectable limit of this assay kit is 0.01 EU/mL.

MATERIALS REQUIRED *(NOT PROVIDED)*

- 25% acetic acid solution to use for Stop Reagent
- Pyrogen-free pipettes, 1 mL, 100 µL, or automatic pipettes with pyrogen-free tips
- Pyrogen-free 96-well microplates or plate strips
- Test tube rack
- Microplate reader with 405 nm filter
- Dry Bath Heater at 37 ± 1 °C with microplate adapter (if using a non-incubating microplate reader)
- Pyrogen-free reagent reservoirs (optional)
- Multi-channel pipettor (optional)
- Vortex mixer
- Timer

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MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

COMPONENT	KIT CATALOG #	QUANTITY PER TEST KIT	CAP COLOR	DESCRIPTION	STORAGE OF MATERIAL
Lyophilized Amebocyte Lysate (AL)	ECT032	1 vial (1.7 mL/vial)	Blue	AL is derived from *Tachypleus tridentatus*.	Store at 2-8 °C. Avoid exposure to temperatures above 25 °C or prolonged bright light. Use reconstituted AL reagent within 10 minutes.
	ECT064	2 vials (1.7 mL/vial)			
Endotoxin-free Water	ECT032	1 vial (50 mL/vial)	Blue	Endotoxin-free Water. Endotoxin concentration <0.005 EU/mL. Endotoxin-free Water is used to rehydrate Endotoxin Control Standard, Amebocyte Lysate, Chromogenic Substrate, dilute Endotoxin Standards and samples, and as a negative control (blank).	Store at 2-30 °C.
	ECT064	2 vials (50 mL/vial)			
Endotoxin Control Standard	ECT032	1 vial	Red	Lyophilized *E. coli* (0111:B4) Endotoxin Standard. Reconstitute Endotoxin Control Standard with Endotoxin-free Water. *Actual potency printed on the vial label.*	Reconstituted Solutions <20 EU/mL are stable for 4 hours at 2-8 °C.
	ECT064	2 vials			
Chromogenic Substrate	ECT032	1 vial	Yellow	Lyophilized Chromogenic Substrate. Each vial of substrate is rehydrated with labeled amount of Endotoxin-free Water.	Store at 2-8 °C. Reconstituted Chromogenic Substrate is stable for one week at 2-8 °C.
	ECT064	2 vials			

SAMPLE COLLECTION AND PREPARATION

All glassware, plastic ware, and diluents contacting samples or reagents must be endotoxin-free. Glassware and other heat-stable apparatus can be depyrogenated in oven using a validated process, a commonly used minimum time and temperature setting is 60 minutes at 250 °C. Store samples under conditions that prevent bacteriological activity. For temporary storage (less than 24 hours), keep samples at 2-8 °C; for long-term storage, keep samples below -10 °C.

The optimal pH range for the Amebocyte Lysate-Endotoxin reaction is 6-8. Adjust acidic or basic samples to this range using endotoxin-free 0.1 N sodium hydroxide, 0.1 N hydrochloric acid, or endotoxin-free Tris buffer. Always measure the pH of an aliquot of the bulk sample to avoid contaminating the main sample with the pH electrode. Test for and eliminate potential interfering substances as described in the Product Inhibition/Enhancement section.

REAGENT PREPARATION

Allow reagents to reach room temperature before use. Prepare all solutions at room temperature.

Preparation of Endotoxin Standards

Note: *Reconstitute Endotoxin Control Standards immediately before use.*

1. Reconstitute Endotoxin Control Standard with Endotoxin-free Water using the volume specified in the Certificate of Analysis. Vortex vigorously for 5 minutes to obtain a 10 EU/mL Endotoxin Stock Solution.

2a. Preparation of High Endotoxin Standard range 0.1-1.0 EU/mL

CONCENTRATION	INSTRUCTIONS
1.0 EU/mL	Mix 0.2 mL of 10 EU/mL Endotoxin Stock Solution with 1.8 mL Endotoxin-free Water, vortex for 1 minute to obtain a 1.0 EU/mL endotoxin solution.
0.5 EU/mL	Mix 1 mL of 1.0 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water, vortex for 1 minute to obtain a 0.5 EU/mL endotoxin solution.
0.25 EU/mL	Mix 0.5 mL of 1.0 EU/mL endotoxin solution with 1.5 mL Endotoxin-free Water, vortex for 1 minute to obtain a 0.25 EU/mL endotoxin solution.
0.1 EU/mL	Mix 0.2 mL of 1.0 EU/mL endotoxin solution with 1.8 mL Endotoxin-free Water, vortex for 1 minute to obtain a 0.1 EU/mL endotoxin solution.
Blank	Use 0.5 mL of Endotoxin-free water.

2b. Preparation of Low Endotoxin Standard range 0.01-0.1 EU/mL

CONCENTRATION	INSTRUCTIONS
1.0 EU/mL	Mix 0.2 mL of 10 EU/mL Endotoxin Stock Solution with 1.8 mL Endotoxin-free Water, vortex for 1 minute to obtain a 1.0 EU/mL endotoxin solution.
0.1 EU/mL	Mix 0.2 mL of 1.0 EU/mL endotoxin solution with 1.8 mL Endotoxin-free Water, vortex for 1 minute to obtain a 0.1 EU/mL endotoxin solution.
0.05 EU/mL	Mix 1 mL of 0.1 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water, vortex for 1 minute to obtain a 0.05 EU/mL endotoxin solution.
0.025 EU/mL	Mix 0.5 mL of 0.1 EU/mL endotoxin solution with 1.5 mL Endotoxin-free Water, vortex for 1 minute to obtain a 0.025 EU/mL endotoxin solution.
0.01 EU/mL	Mix 0.2 mL of 0.1 EU/mL endotoxin solution with 1.8 mL Endotoxin-free Water, vortex for 1 minute to obtain a 0.01 EU/mL endotoxin solution.
Blank	Use 0.5 mL of Endotoxin-free water.

3. Discard the remaining endotoxin dilutions.

Preparation of AL Reagent:

1. Reconstitute Lyophilized Amebocyte Lysate (AL) immediately before use.
2. Gently tap the vial to settle the powder at the bottom.
3. Add 1.7 mL of Endotoxin-Free Water to the vial.
4. Mix gently by tilting and swirling until the contents are fully dissolved. Avoid vortexing and foaming.
5. After thawing, the reconstituted AL Reagent is for single use only and should be gently swirled to mix before use.
   Note: *Undiluted serum interferes with the assay and must be diluted 50-100X, cleared of red blood cells, and may require heat shocking treatment at 70 °C. To inhibit bacterial activity, store samples at 2-8 °C for 24 hours or at -20 °C for longer periods.*

Preparation of Chromogenic Substrate:

Reconstitute lyophilized substrate vial with 3.4 mL Endotoxin-free Water to prepare substrate solution.
Note: *The reconstituted Chromogenic Substrate is stable for one week at 2-8 °C.*

Preparation of Stop Reagent:

Mix the specified volumes of Endotoxin-free Water and acetic acid to prepare 25% acetic acid Stop Reagent.

Endotoxin-free Water (mL)	Acetic acid, 99.8% (mL)	Stop Reagent (mL)
15	5	20
30	10	40

TEST PROCEDURE

The following steps must be performed at 37 °C ± 1 °C. Preheat the microplate for 10 minutes on the microplate reader or dry bath heater before adding reagents.

For detection of endotoxin in the range of 0.01-0.1 EU/mL:

1. Pre-equilibrate microplate at 37 °C ± 1 °C in the heating block adapter.
2. Maintain microplate at 37 °C ± 1 °C, dispense 50 µL of Endotoxin-free Water (as blanks), 0.01 EU/mL, 0.025 EU/mL, 0.05 EU/mL, 0.1 EU/mL Endotoxin Standard Solutions, and test samples into the designated wells. Run all reactions in duplicate.
3. Dispense 50 µL of AL Reagent into each well. (If using a multi-channel pipettor, transfer an adequate amount of AL Reagent into a reagent reservoir, then dispense 50 µL into each well.) Mix immediately and thoroughly. Maintain consistency in the order of reagent addition and pipetting across all wells and rows.
4. Place microplate cover on the plate and incubate in the heating block for the time (T1) specified in the Certificate of Analysis for curve range 0.01-0.1 EU/mL.
5. Transfer an adequate amount of Chromogenic Substrate Solution into a reagent reservoir (optional). Dispense 100 µL of the substrate solution into each well and mix immediately. Maintain the same addition order as in Step 3.
6. Cover the microplate and incubate for the time (T2) specified in the Certificate of Analysis for curve range 0.01-0.1 EU/mL.
7. Transfer an adequate amount of Stop Reagent (25% acetic acid solution) into a reagent reservoir (optional). After incubation time in Step 6, dispense 50 µL of Stop Reagent into each well, maintaining the same pipetting order as in Steps 3 and 5. Mix immediately and thoroughly.
8. Measure the absorbance of each well at 405 nm.

The table below summarizes the test procedure.

	Endotoxin Standard Wells	Sample Wells	Blank Wells
Pre-equilibrate the microplate at 37 °C ± 1 °C.			
Add Endotoxin-free Water			50 µL
Add Endotoxin Standard	50 µL		
Add Sample		50 µL	
Add AL Reagent	50 µL	50 µL	50 µL
Mix well, incubating at 37 °C ± 1 °C for the time (T1) indicated in Certificate of Analysis.			
Add Chromogenic Substrate Solution	100 µL	100 µL	100 µL
Mix well, incubating at 37 °C ± 1 °C for the time (T2) indicated in Certificate of Analysis.			
Add 50 µL of Stop Reagent	50 µL	50 µL	50 µL
Mix well, read the absorbance at 405 nm			

For detection of endotoxin ranging from 0.1-1.0 EU/mL:

All test procedures are identical to those used for the 0.01-0.1 EU/mL range, except that in Step 2 the Endotoxin Standard Solutions are prepared at 0.1, 0.25, 0.5, and 1 EU/mL, and in Steps 4 and 6 the incubation times are adjusted according to the Certificate of Analysis, using T1 for Step 4 and T2 for Step 6.

DATA COLLECTION AND ANALYSIS

1. Obtain the absorbance values at 405 nm.
2. Construct the Standard Curve

   Y = a X + b, where

   Y = absorbance, X = endotoxin concentration,

   a = slope of the regression curve, b = the Y intercept.

ROUTINE TESTING

For routine testing, prepare a series of endotoxin standards and assay them alongside the unknown samples under the same conditions. Determine the endotoxin concentration in the unknowns by comparing performance to the standards. Each standard and unknown sample should be tested in at least duplicate, and the average absorbance of all replicates should be used for calculations. A Positive Product Control may be included to monitor for product inhibition or enhancement.

INITIAL QUALIFICATION

As recommended in the Pharmacopeia, validation of the Amebocyte Lysate Endotoxin assay should be performed when conditions that are likely to influence the test result change.

Standard Curve Validation. Upon receipt of a new lot of AL Reagent, a standard curve validation test must be performed. Prepare at least three endotoxin concentrations within the standard curve range indicated by the Certificate of Analysis. Perform the assay using at least three replicates of each standard endotoxin concentration. Unlike the routine testing, do not average the absorbances of all replicates. The absolute value of the correlation coefficient (r) must be ≥ 0.980 for the endotoxin concentration range tested.

Test for Interfering Factors. The test for interfering factors must be repeated whenever conditions that could affect the test results change. This includes, but is not limited to, changes in test sample formulations or the AL Reagent provider. Refer to the Product Inhibition/Enhancement section for details.

PERFORMANCE CHARACTERISTICS

The test is valid only when all of the following requirements met.

1. Prepare at least three endotoxin concentrations within the concentration range along with blanks to generate the standard curve. Perform the assay using at least two replicates of each standard endotoxin concentration.
2. The absolute value of the correlation coefficient (r) of the calculated standard curve should be ≥ 0.980.
3. Both absorbances of the negative controls are lower than that of the lowest concentration of the endotoxin standard curve.
4. The %C.V. of replicate absorbances should be less than 10%. To calculate the coefficient of variation (C.V.), divide the standard deviation of the absorbances by the mean and express as a percentage.

PRODUCT INHIBITION/ENHANCEMENT

If there is potential that the sample contains interfering substances, recovery rate test should be run. Prepare a Positive Product Control, which is a sample of the product spiked with a known amount of endotoxin. The spike concentration of endotoxin (λm) should be in the middle of the standard curve range.

1. Analyze the spiked sample (PPC) along with the unspiked sample.
2. Determine the endotoxin concentration in spiked test sample (Cs) and in unspiked test sample (Ct).
3. Calculate the recovery rate (R): $R = (Cs-Ct) / \lambda m \times 100\%$.
4. Recovery rates within the range of 50-200% suggest non-significant interference. Recovery rates out of the range of 50-200% suggest significant interference. Dilution and modification are commonly employed to reduce the interference to a non-significant level.
5. If R is outside the 50-200% range, repeat the inhibition test using a series of test sample dilutions, ensuring that the dilution does not exceed the Maximum Valid Dilution.
6. For routine endotoxin concentration assays, it is best to select a test sample dilution at which R is closest to 100%.

COLORED SAMPLES

A sample blank should be run for colored samples, and samples which produce yellow product in acidic environments. The only difference between sample blank and sample is replacing AL Reagent with equal volume of Endotoxin-free Water for sample blank. If the absorbance of the sample blank is > 0.5, the sample should be diluted and reanalyzed.

REFERENCES

- FDA (1987) Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test.
- United States Pharmacopeial Convention. General Chapter <85> Bacterial Endotoxins Test. United States Pharmacopeia (USP).
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