A highly sensitive assay for endotoxin detection and quantitation for a variety of sample types

Endotoxin contamination is a common problem with recombinant proteins and nucleic acids purified from gram-negative bacteria such as *E. coli*. Endotoxins are lipopolysaccharides (LPS), which are heat-stable molecules associated with the outer membranes of certain gram-negative bacteria. When bacterial cells are actively growing or when their membranes disintegrate upon death, the essential LPS components of the cell wall are released into the surrounding environment. Lipopolysaccharides are large molecules ranging in size from 3,000 to 4,000 Da, consisting of a hydrophobic lipid group covalently bound to a long complex polysaccharide tail (Figure 1). Endotoxin contamination is dangerous and can trigger endotoxic shock, inflammation, or sepsis in animals and tissue culture.

In the early 1950s, Frederick Bang discovered that the blood cells (amebocytes) of the horseshoe crab clot in the presence of low levels of endotoxins. The gel-clot method established from this observation is an economical, specific, and sensitive method to detect endotoxins. The use of the amebocyte lysate has become the industry standard to detect endotoxins, in the pharmaceutical and food industries as well as life science and medical research.

Recognizing the need for a sensitive endotoxin detection method, Thermo Fisher Scientific has extended its endotoxin detection line to include the Thermo Scientific™ Pierce™ Chromogenic Endotoxin Quant Kit, which accurately detects endotoxins at levels as low as 0.01 EU/mL in samples. The Pierce Chromogenic Endotoxin Quant Kit is an endpoint amebocyte lysate assay that accurately detects and quantitates endotoxins (lipopolysaccharides) in a variety of sample types, including proteins, peptides, nucleic acids, and antibodies.

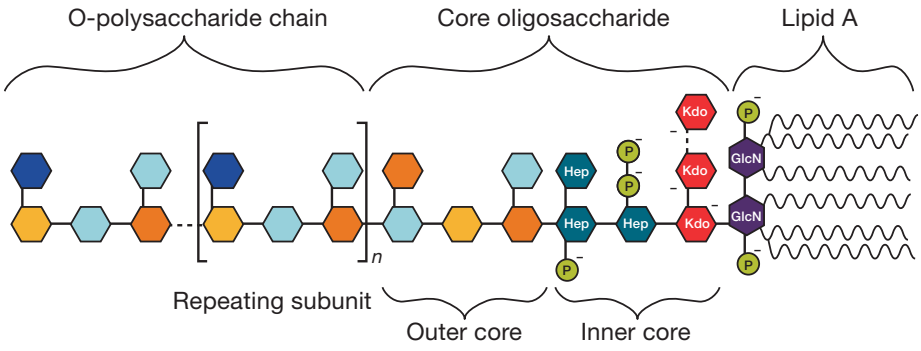

Figure 1. Structure of lipopolysaccharide. Endotoxins are complex lipopolysaccharides, which are biologically active structural components of the outer cell membrane of gram-negative bacteria. They consist of a core oligosaccharide chain, O-specific polysaccharide side chain (O-antigen), and a lipid component, lipid A, which is responsible for the toxic effects.

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The principle of the assay is based on the activation of factor C, factor B, and pro–clotting enzyme in the amebocyte lysate in the presence of endotoxin. The amount of endotoxin is quantitated by the addition of a chromogenic substrate, Ac-Ile-Glu-Ala-Arg-pNA. The endotoxin-activated pro–clotting enzyme catalyzes the release of p-nitroaniline (pNA) to produce a yellow color (Figure 2). After the reaction is stopped, the released pNA is photometrically measured at 405 nm (Figure 3). The developed color intensity is directly proportional to the amount of endotoxin present in the sample and is calculated using a standard curve. Here we present data demonstrating the performance of Pierce endotoxin quantitation kits.

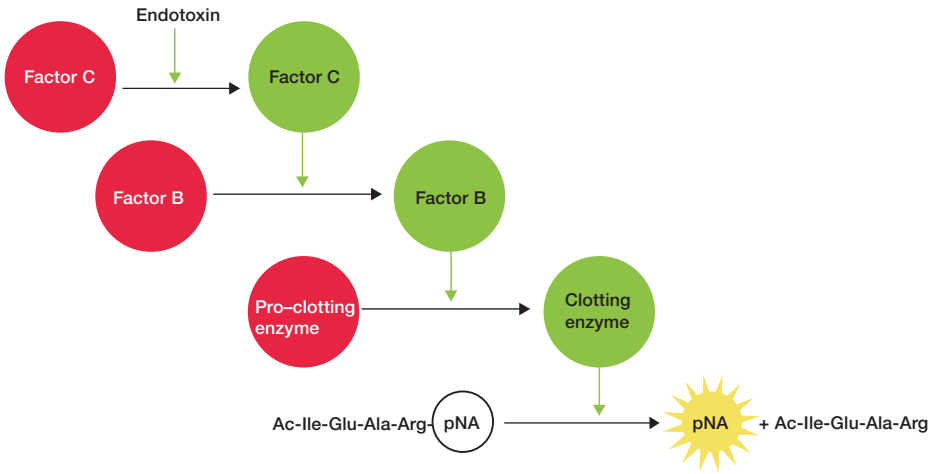


Figure 2. Coagulation cascade in horseshoe crab blood. LPS (endotoxin) activates plasma membrane–bound factor C. The activated factor C activates factor B, which activates the clotting enzyme that triggers the exocytotic release of the clotting cascade. Upon addition of a chromogenic substrate, Ac-Ile-Glu-Ala-Arg-pNA, the activated protease, clotting enzyme, catalyzes the release of p-nitroaniline (pNA), resulting in a yellow color that can be quantitated by measuring the absorbance at 405 nm and extrapolating from a standard curve. Red indicates inactive enzymes, and green indicates active enzymes.

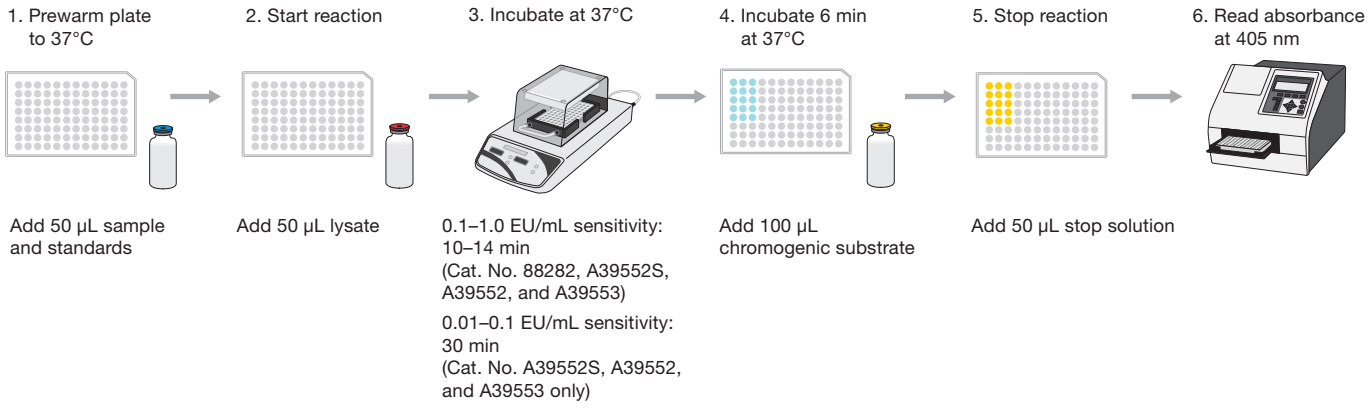


Figure 3. Assay protocol for Pierce endotoxin quantitation kits.

Sensitivity and reproducibility

Sensitive endotoxin testing is essential because of the sample limitations and low endotoxin levels required for cell culture and animal research. The Pierce Chromogenic Endotoxin Quant Kit offers high sensitivity and reproducibility with two linear dynamic ranges of 0.01–0.1 EU/mL and 0.1–1.0 EU/mL (Figure 4). The test-to-test and operator-to-operator reproducibility of this endpoint amebocyte lysate assay resulted in a coefficient of variation (CV) of 3%. The lower sensitivity range permits higher dilution of samples that are limited in quantity or contain interfering substances, helping to avoid potential false negatives or false positives in endotoxin quantitation.

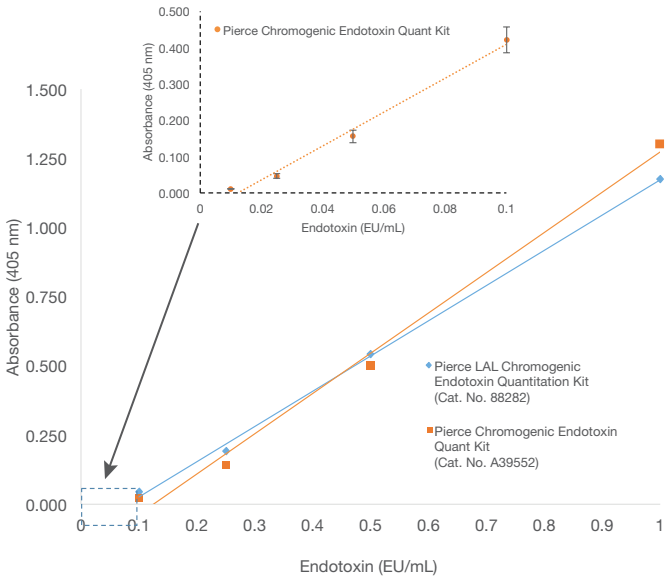


Figure 4. Standard curves for Pierce endotoxin quantitation kits. The standard curves show exceptional linearity with $r^2 = 0.99$. The Pierce Chromogenic Endotoxin Quant Kit has a lower-range standard curve of 0.01–0.1 EU/mL. The standard curve of 0.1–1.0 EU/mL represents a 14-minute incubation with endotoxin standards and amebocyte lysate, followed by a 6-minute incubation with the chromogenic substrate. For the lower range, a 30-minute incubation with endotoxin standards and amebocyte lysate was followed by a 6-minute incubation with the chromogenic substrate. Consistency and reproducibility (n = 17; CV = 3%) is shown for the low-range standards (0.01–0.1 EU/mL).

Compatibility

Amebocyte lysate assays can be affected by many factors that can cause inhibition or enhancement leading to false negatives or false positives. Factors that can lead to inhibition of the amebocyte lysate assay include reaction temperature, sample pH, ionic strength, and metal ions (e.g., magnesium and calcium). Serum proteins, nucleic and fatty acids, surfactants, and chelating reagents (e.g., EDTA and heparin) cause changes in molecular structure of endotoxin aggregates that can result in inaccurate or total inhibition of the amebocyte lysate assay. In addition, surfactants (e.g., Triton™ X-100, SDS, deoxycholate) used in various protein workflows alter the supramolecular structure of lipopolysaccharides and therefore can interfere with their detection and quantitation. If any of these interfering substances are present in test samples, the samples need to be diluted. Table 1 summarizes compatibility levels of common reagents with the amebocyte lysate assay. To be considered free of interfering factors in the test, the measured concentration of endotoxin added to the sample must be within 50%–200% of the known added amount.

Table 1. Highest acceptable concentration of typical reagents for valid endotoxin spike recovery using the Pierce Chromogenic Endotoxin Quant Kit. Concentrations listed refer to the actual concentration in the sample that produced no decrease in quantitation values when spiked with 0.5 EU endotoxin. Dilutions are expressed in the form of a ratio, where 1:100 means a 100-fold dilution.

Test sample	Highest acceptable concentration of reagent for valid endotoxin spike recovery
Complete* Ham's F-12K (Kaighn's) Medium supplemented with 1% fetal bovine serum	1:100
Complete* Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% fetal bovine serum	1:100
DNA**	0.01 µg/mL
Human serum albumin	<1 µg/mL
Glycerol	1%
Sucrose	1%
D-glucose (dextrose)	1%
Triton X-100	1%
SDS	0.001%
Tween™-20	0.05%
EDTA (pH 6.0)	10 mM
EDTA (pH 8.0)	0.4 mM
Heparin (1,000 IU)	1:500
β-glucan†	10 ng/mL

* Supplemented with Penicillin-Streptomycin-Glutamine (100X), diluted to 1X and contains phenol red.
** Sheared salmon sperm DNA.
† Highest level tested that exhibited no enhancement.

Another common interfering substance in the amebocyte lysate assay is (1,3)-β-D-glucan, a cell wall component of bacteria and fungi. Although β-glucan is not pyrogenic, it can activate factor G, which is able to trigger the coagulation cascade, producing false positives in the assay (Figure 5). The Pierce Chromogenic Endotoxin Quant Kit (Cat. No. A39552) is compatible with β-glucans, and in samples containing ≤10 ng/mL of (1,3)-β-D-glucan, no enhancement is exhibited. The Pierce LAL Chromogenic Endotoxin Quantitation Kit (Cat. No. 88282) is not compatible with β-glucans, and requires blocking with a β-glucan blocker to avoid enhancement effects (false positives) from β-glucan contamination.

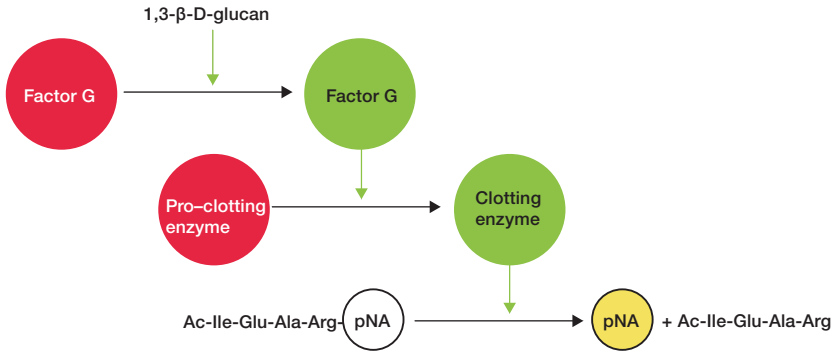
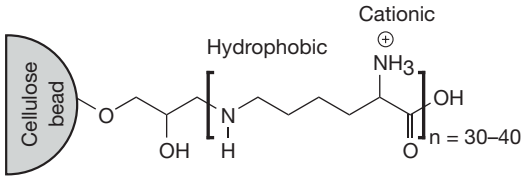


Figure 5. Activation of the clotting enzyme by (1,3)-β-D-glucan. The presence of β-glucans activates a pathway that is independent of the amebocyte lysate response to endotoxins resulting in false-positive determination of bacterial endotoxins in the sample. The Pierce Chromogenic Endotoxin Quant Kit is resistant to β-glucans. Red indicates inactive enzymes, and green indicates active enzymes.

Determining efficiency of endotoxin removal

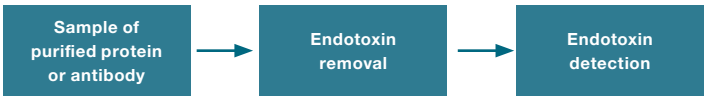
Endotoxin-contaminated protein or antibody samples transfected into cells or injected into an animal host can initiate a strong immune response, resulting in systemic inflammatory response syndrome (SIRS) and/or sepsis. Elimination of endotoxins from samples produced from gram-negative bacteria prior to cell transfection or animal injection is a necessity. Ultrafiltration, polymixin B affinity resin, or resin- or membrane-based chromatography are the traditional methods of endotoxin removal. These methods have limitations in protein recovery or endotoxin binding capacity, or have toxicity concerns. Thermo Fisher Scientific offers a modified ε-poly-L-lysine [poly(ε-lysine)] affinity resin (nontoxic polymer of the natural amino acid lysine) that displays both excellent endotoxin binding capacity and protein recovery. The polylysine affinity ligands bind endotoxins through both ionic and hydrophobic interactions (Figure 6). With a 2,000,000 EU/mL binding capacity, endotoxin levels can be reduced by 99% in samples containing initial endotoxin levels of 10,000 EU/mL. Typical protein samples processed with the Thermo Scientific™ Pierce™ High Capacity Endotoxin Removal Resin have a final endotoxin concentration below 5 EU/mL (Figure 7B). A general workflow of endotoxin removal from purified sample to endotoxin detection is presented in Figure 7A.



Poly(ε-lysine) resin
Pierce High Capacity Endotoxin Removal Resin

Figure 6. The poly(ε-lysine) affinity ligand binds endotoxins through both ionic and hydrophobic interactions. The multiple ε-aminobutyl groups impart both a positive charge via the primary amines and a hydrophobic characteristic via the butyl spacer between primary amines. The hydrophilic nature of the porous cellulose base matrix is masked by thorough derivatization of its interior and exterior surfaces with the poly(ε-lysine) ligand.

A



B

Protein	Initial endotoxin concentration (EU/mL)	Final endotoxin concentration (EU/mL)	Endotoxin removal
His-tag GFP isolated from *E. coli*	9,780	1	99%
Bovine serum albumin*	10,000	5	99%

* BSA (1.25 µg/mL) was spiked with 10,000 EU/mL.

Figure 7. Efficient removal of endotoxins by the Pierce High Capacity Endotoxin Removal Resin. (A) General workflow for endotoxin removal and quantitation. **(B)** Determination of final endotoxin concentrations in purified protein samples in preparation for downstream applications.

Conclusion

The Pierce Chromogenic Endotoxin Quant Kit (Cat. No. A39552) and the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Cat. No. 88282) are sensitive endpoint assays for the detection and quantitation of endotoxins from a variety of sample types. The high sensitivity of the Pierce Chromogenic Endotoxin Quant Kit allows for sample conservation as well as determination of endotoxin levels for verification of safe sample use in downstream applications such *in vivo* or cell culture transfection studies.

The Pierce High Capacity Endotoxin Removal Resin is effective in eliminating endotoxins from samples containing proteins of various sources, sizes, and charges. The high binding capacity and low protein retention make this resin suitable for many protein sample types, including antibodies.

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Ordering information

Product	Quantity	Cat. No.
Pierce LAL Chromogenic Endotoxin Quantitation Kit	50 reactions	88282
Pierce Chromogenic Endotoxin Quant Kit	30 reactions	A39552S
	60 reactions	A39552
	240 reactions	A39553
Pierce High Capacity Endotoxin Removal Resin	10 mL	88270
	100 mL	88271
	250 mL	88272
Pierce High Capacity Endotoxin Removal Spin Columns	0.25 mL capacity	88273
	0.5 mL capacity	88274
	1 mL capacity	88276

References

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