INSTRUCTIONS

Pierce LAL Chromogenic Endotoxin Quantitation Kit

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88282

Number	Description
88282	**Pierce LAL Chromogenic Endotoxin Quantitation Kit,** sufficient reagents to perform 50 assays of standards and samples in a microplate (i.e., 50 wells total)
	Kit Contents:
	***Escherichia coli* (*E. coli*) Endotoxin Standard (011:B4),** lyophilized, 1 vial, 15-40 endotoxin units (EU)/mL upon reconstitution
	Limulus Amebocyte Lysate (LAL), lyophilized, 2 vials, 1.4mL/vial upon reconstitution
	Chromogenic Substrate, lyophilized, 1 vial, 6.5mL/vial upon reconstitution
	Endotoxin-free Water, 1 vial, 30mL
	Storage: Upon receipt store at 4°C. Product shipped with an ice pack.

Introduction

The Thermo Scientific™ Pierce™ LAL Chromogenic Endotoxin Quantitation Kit is an efficient, quantitative endpoint assay for the detection of gram-negative bacterial endotoxins. Bacterial endotoxin catalyzes the activation of a proenzyme in the modified Limulus Amebocyte Lysate (LAL). The activated proenzyme then catalyzes the splitting of *p*-Nitroaniline (pNA) from the colorless substrate, Ac-Ile-Glu-Ala-Arg-pNA; the activation rate is proportional to the sample endotoxin concentration. After stopping the reaction, the released pNA is photometrically measured at 405-410nm. The correlation between absorbance and endotoxin concentration is linear in the 0.1-1.0EU/mL range. The developed color intensity is proportional to the amount of endotoxin present in the sample and can be calculated using a standard curve.

Important Product Information

- Accurate pipetting is critical for maintaining consistent results. A repetitive pipettor can aid in normalizing volumes between samples. Ensure pipetting order and rate of reagent addition remain consistent from well to well and row to row.
- All materials (e.g., pipette tips, glass tubes, microcentrifuge tubes and disposable 96-well microplates) must be endotoxin-free.
- Adjust the sample pH to 6-8 using endotoxin-free 0.1M NaOH or 0.1M HCl. Avoid pH-electrode contamination of the sample by testing the pH of a small sample taken from the bulk sample.
- Components of undiluted serum interfere in the assay. Serum samples must be diluted 50- to 100-fold to be compatible. The serum must be completely free of RBCs, and the diluted sample may need to be heat-shocked (70°C for 15 minutes).
- To stop all bacteriological activity, store samples to be tested at 2-8°C for <24 hours or -20°C for >24 hours.
- Maintaining the correct temperature is critical for reproducibility. Use a proper heating block at 37°C±1°C. Do not use a cabinet-style incubator to perform the assay.
- Endotoxin adheres to glass and plastic surfaces; before pipetting, vortex solutions to ensure the correct endotoxin concentrations are measured.
- Glass tubes are preferred for making standard stock solutions; however, polystyrene or polypropylene microcentrifuge tubes (1.5mL) may also be used. When using microcentrifuge tubes, dedicate the bag of tubes for the assay and follow aseptic techniques.
- If the test sample endotoxin concentration is >1.0EU/mL, dilute the sample five-fold in endotoxin-free water. Re-test.

- Assay inhibition occurs when substances in the test sample interfere with the LAL reaction. In the chromogenic assay, this inhibition results in a lower final absorbance, indicating lower levels of endotoxin than what may be present in the test sample. Determine the lack of product inhibition for each sample undiluted or at an appropriate dilution. See the Additional Information Section for more details.
- Some LAL-reactive glucans (LRGs) may result in false positive signal in the LAL assay.[1] In samples where LAL-reactive glucans may be present, researchers may want to compare cellulase-treated samples to untreated samples to determine contribution of signal resulting from LRGs.
- Samples turning yellow after addition of the Stop Reagent (25% acetic acid) or possessing significant initial color may require special attention. See the Additional Information Section for more details.
- The kit reagents are "matched" to comply with Food and Drug Administration (FDA) requirements for endotoxin testing. Each LAL lot is tested for functionality using the United States Reference Standard EC-6. The LAL lot is then matched to a lot of our Control Standard Endotoxin (CSE) by testing in parallel with the Reference Standard Endotoxin (RSE). The RSE/CSE correlation assay determines the potency of each CSE lot when used with each matching LAL lot.

Additional Materials Required

- Disposable endotoxin-free glass tubes or 1.5mL microcentrifuge tubes
- Disposable endotoxin-free pipette tips
- Disposable endotoxin-free 96-well microplates
- Heating block at 37°C±1°C
- Pipettor
- Repetitive pipettor (optional)
- Microplate reader
- 25% acetic acid (Stop Reagent)

Material Preparation

Note: Equilibrate all reagents to room temperature before use.

A. Endotoxin Standard Stock Solutions

1. Each *E.coli* Endotoxin Standard vial contains ~15-40EU of lyophilized endotoxin; the actual vial concentration is printed on the label. Reconstitute by adding 1mL of room temperature endotoxin-free water to make Endotoxin Standard Stock. For example, a vial with a concentration of 26EU, when reconstituted with 1.0mL of endotoxin-free water, will yield a concentration of 26EU/mL. Vortex the solution vigorously for at least 15 minutes on a high speed vortex mixer before use.

   Note: Store lyophilized *E.coli* Endotoxin Standard at 2-8°C. Reconstituted stock solution is stable for 4 weeks at 2-8°C. Prior to subsequent use, warm the solution to room temperature and vigorously mix for 15 minutes. (This is important since the endotoxin adheres to sides of the glass vial.)
2. Prepare Standard Stock Solutions from the Endotoxin Standard Stock using the dilutions and procedures in Table 1.

Table 1. Dilutions and procedures for preparing Standard Stock Solutions.

Vial	Volume of Endotoxin Standard Stock (mL)	Volume of vial A (mL)	Endotoxin-free Water (mL)	Final endotoxin concentration (EU/mL)
A	0.05	–	(X-1)/20*	1.00
B	–	0.25	0.25	0.50
C	–	0.25	0.75	0.25
D	–	0.10	0.90	0.10

*X = endotoxin concentration of the *E.coli* Endotoxin Standard supplied with the kit; refer to the Certificate of Analysis to get the lot-specific concentration.

A. Prepare a solution containing 1.0EU/mL of endotoxin standard by diluting 0.05mL of the Endotoxin Standard Stock with [(X-1)/20]mL of endotoxin-free water, where X equals the endotoxin concentration of the vial (e.g., if X= 26EU/mL, then dilute 0.05mL of this stock with (26-1)/20, or 1.25mL, of endotoxin-free water). **Vigorously vortex the solution for ≥1 minute before proceeding**.

B. Transfer 0.25mL of the 1.0EU/mL Standard vial A into a tube containing 0.25mL of endotoxin-free water to prepare 0.5EU/mL Standard Stock Solution and **vortex the solution vigorously for 1 minute before use.**

C. Transfer 0.25mL of 1.0EU/mL Standard vial A into a tube containing 0.75mL of endotoxin-free water to prepare 0.25EU/mL Standard Stock Solution and **vortex the solution vigorously for 1 minute before use.**

D. Transfer 0.1mL of 1.0EU/mL Standard vial A into a tube containing 0.90mL of endotoxin-free water to prepare 0.1EU/mL Standard Stock Solution and **vortex the solution vigorously for 1 minute before use.**

B. Limulus Amebocyte Lysate (LAL)

1. The LAL reagent contains lyophilized lysate prepared from the circulating amebocytes of the horseshoe crab *Limulus polyphemus.* **Reconstitute immediately before use** with 1.4mL of endotoxin-free water and swirl gently to dissolve the powder. If more than one vial is required, pool two or more vials before use. **Avoid foaming; do not vortex the solution**.

   Note: Store lyophilized LAL protected from light at 2-8°C. Reconstituted LAL reagent is stable for 1 week at -20°C or colder if frozen immediately after reconstitution. Upon thawing, the reconstituted LAL may be used only one time; once thawed, gently swirl the reagent to mix before adding to samples.

C. Chromogenic Substrate

1. Each vial contains ~7mg of lyophilized substrate. Reconstitute the Chromogenic Substrate by adding 6.5mL of endotoxin-free water to yield a final concentration of ~2mM.

   Note: Store lyophilized Chromogenic Substrate protected from light at 2-8°C. Reconstituted Chromogenic Substrate is stable for 4 weeks when stored at 2-8°C. **Prior to use, warm up sufficient substrate solution for the assay to 37°C±1°C.**

Microplate Assay Procedure

Note: Equilibrate all reagents to room temperature before use.

1. Pre-equilibrate the microplate in a heating block for 10 minutes at 37°C±1°C.

   Note: Do not use cabinet-style incubator to perform the test.

2. With the microplate maintained at 37°C±1°C, carefully dispense 50µL of each standard or unknown sample replicate into the appropriate microplate well.

   Note: Each series of determinations must include duplicate runs of a blank and the four endotoxin standards; the blank contains 50µL of endotoxin-free water. If reaction inhibition is possible, see the Additional Information Section.

3. At time T=0, add 50µL of LAL reagent to each well using a pipettor. Begin timing as the LAL is added. Once the LAL has been added into all plate wells, briefly remove from the heating block and gently tap several times to facilitate mixing. Cover the plate with the lid and return to heating block to incubate at 37°C±1°C for 10 minutes.

   Note: Ensure pipetting order and rate of reagent addition remain consistent from well-to-well and row-to-row.

4. After exactly T=10 minutes, add 100µL of Chromogenic Substrate solution (prewarmed to 37°C±1°C) to each well. Once the substrate solution has been added into all plate wells, briefly remove from the heating block and gently tap several times to facilitate mixing. Cover the plate with lid and return to heating block to incubate the plate at 37°C±1°C for 6 minutes.

   Note: Pipette the substrate solution in the same manner as in step 3. Maintain a consistent pipetting speed.

5. At T=16 minutes, add 100µL of Stop Reagent (25% acetic acid). Once the stop reagent was added into all plate wells, remove the plate from heating block and gently tap several times to facilitate mixing.

   Note: Maintain the same pipetting order as in steps 3 and 4.

6. Measure the absorbance at 405-410nm on a plate reader.

7. Subtract the average absorbance of the blank replicates from the average absorbance of all individual standards and unknown sample replicates to calculate mean Δ absorbance.

8. Prepare a standard curve by plotting the average blank-corrected absorbance for each standard on the y-axis vs. the corresponding endotoxin concentration in EU/mL on the x-axis. The coefficient of determination, r^2, must be ≥0.98.

9. Use the formulated standard curve (linear regression) to determine the endotoxin concentration of each unknown sample (Figure 1).

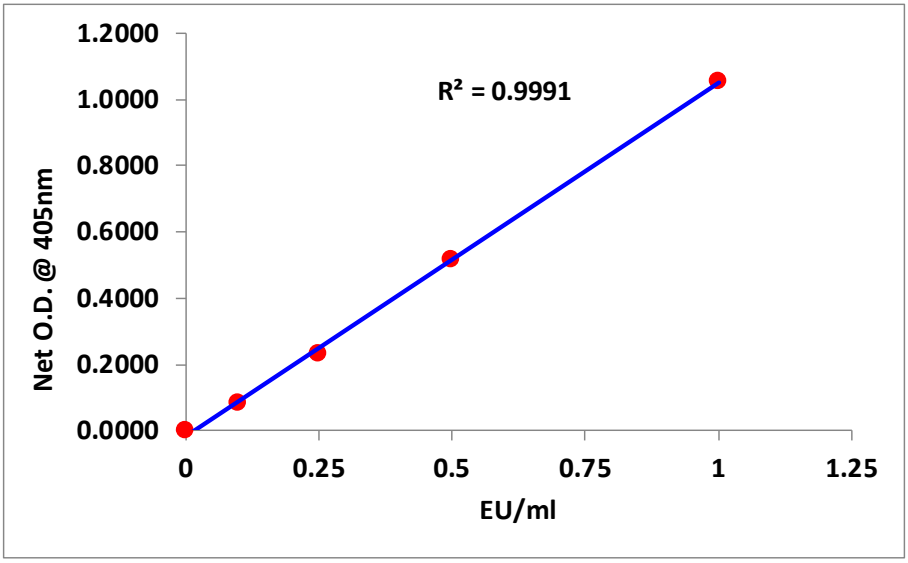

Figure 1. Example standard curve for the quantitation of endotoxin in a chromogenic assay.

Troubleshooting

Problem	Possible Cause	Solution
Non-linear standard curve	Standard Stock Solutions were not mixed well	Vortex the Endotoxin Standard Stock for 15 minutes before each use.
		Vortex all Standard Stock Solutions for 1 minute before each use.
	Pipetting order and rate of reagent addition was irregular	Ensure pipetting order and rate of reagent addition remain consistent from well to well and row to row.
		Use a repetitive pipettor.
	Incubation times were not followed	Strictly adhere to the incubation times.
		Start the timer at the point of adding reagent into the first well.
Higher absorbance in blank than standards	Materials (e.g., tips, vials, microplates) were contaminated	Use endotoxin-free materials.

Additional Information

A. Product Inhibition/Enhancement

Product inhibition occurs when substances in the test sample interfere with the LAL reaction. In the chromogenic assay, this inhibition results in a lower, final absorbance, indicating lower levels of endotoxin than what may be present in the test sample. Determine the lack of product inhibition for each sample undiluted or at an appropriate dilution.

To verify the lack of product inhibition, spike an aliquot or dilution of a test sample with a known amount of endotoxin (e.g., 0.5EU/mL). Assay the spiked sample and the unspiked samples to determine the respective endotoxin concentrations. The difference between the two calculated endotoxin values should equal the known concentration of the spike ±25%.

B. Example for Determination of a Non-inhibitory Dilution

Table 2. Example study for determining inhibition of a sample.

Sample dilution	Spiked[§] concentration (EU/mL)	Unspiked concentration (EU/mL)	Difference
1:10	0.28	0.18	0.10 = Inhibitory
1:20	0.36	0.11	0.25 = Inhibitory
1:40	0.50	< 0.1	0.50 = Non-inhibitory

[§] Spiked concentrations should all show a value of 0.50EU/mL The values of 0.28 and 0.36 are indicative of inhibition at the respective dilutions.

Samples showing inhibition on the LAL reaction may require further dilution to overcome the inhibitory effects. Once the non-inhibitory dilution is determined, the exact dilution can be found by testing two-fold dilutions near that dilution. The degree of inhibition or enhancement is dependent on the product concentration.

Beta glucans are polymers of D-glucose found in fungi and plant cell walls with >1000-fold less LAL reactivity than lipopolysaccharides. Inhibition and false-positive colors can occur in samples contaminated with beta glucans. Use appropriate beta glucan blockers if any contamination is possible.

C. Colored Samples

In the chromogenic assay, samples turning yellow after addition of the Stop Reagent or possessing significant initial color (e.g., tissue culture media) may require special attention. To determine if a sample's intrinsic color will alter the absorbance readings, construct a mock reaction tube by adding 50µL of sample, 150µL of endotoxin-free water and 50µL of Stop Reagent with no incubation. Read the absorbance at 405-410nm. If the absorbance is significantly greater than the absorbance of endotoxin-free water, then the intrinsic color will alter the correct sample absorbance readings. In such cases, include appropriate controls in the assay.

Related Thermo Scientific Products

88270	**Pierce High Capacity Endotoxin Removal Resin,** 10mL
88271	**Pierce High Capacity Endotoxin Removal Resin,** 100mL
88272	**Pierce High Capacity Endotoxin Removal Resin,** 250mL
88273	**Pierce High Capacity Endotoxin Removal Spin Column, 0.25mL,** 5 columns
88274	**Pierce High Capacity Endotoxin Removal Spin Column, 0.50mL,** 5 columns
88276	**Pierce High Capacity Endotoxin Removal Spin Column, 1mL,** 5 columns
20339	**Detoxi-Gel™ Endotoxin Removing Gel**
20344	**Detoxi-Gel Endotoxin Removing Columns**
89896	**Pierce Centrifuge Columns, 2mL,** 25/pkg
89897	**Pierce Centrifuge Columns, 5mL,** 25/pkg
89898	**Pierce Centrifuge Columns, 10mL,** 25/pkg
23225	**Pierce BCA Protein Assay Kit**
22660	**Pierce 660nm Protein Assay Kit**

Reference

[1]Roslansky, P.F. and Novitsky, T.J. (2016). Sensitivity of Limulus amebocyte lysate (LAL) to LAL-reactive glucans. *J Clin Microbiol* **54** (5). Jcm.asm.org/content/29/11/2477.short