Pierce™ Chromogenic Endotoxin Quant Kit

Catalog Numbers A39552S, A39552, and A39553

Doc. Part No. 2162713 **Pub. No.** MAN0017902 **Rev.** A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Contents

Product	Cat. No.	Contents	Storage
Pierce™ Chromogenic Endotoxin Quant Kit	A39552S	Kit sufficient for 30 tests of standards and samples in a microplate Contents: Lyophilized *E. coli* (0111:B4) Endotoxin Standard, 1 vial, 10-50 endotoxin units (EU)/vial Lyophilized Amebocyte Lysate, 1 vial, 1.7 mL/vial upon reconstitution Lyophilized Chromogenic Substrate, 1 vial, 3.4 mL/vial upon reconstitution Endotoxin-Free Water, 1 vial, 50 mL	Store at 4°C.
	A39552	Kit sufficient for 60 tests of standards and samples in a microplate Contents: Lyophilized *E. coli* (0111:B4) Endotoxin Standard, 2 vials, 10-50 endotoxin units (EU)/vial Lyophilized Amebocyte Lysate, 2 vials, 1.7 mL/vial upon reconstitution Lyophilized Chromogenic Substrate, 2 vials, 3.4 mL/vial upon reconstitution Endotoxin-Free Water, 2 vials, 50 mL/vial	
	A39553	Kit sufficient for 240 tests of standards and samples in a microplate Contents: Lyophilized *E. coli* (0111:B4) Endotoxin Standard, 8 vials, 10-50 endotoxin units (EU)/vial Lyophilized Amebocyte Lysate, 8 vials, 1.7 mL/vial upon reconstitution Lyophilized Chromogenic Substrate, 8 vials, 3.4 mL/vial upon reconstitution Endotoxin-Free Water, 4 vials, 50 mL/vial	

Product description

The Thermo Scientific™ Pierce™ Chromogenic Endotoxin Quant Kit is an efficient, quantitative endpoint assay that uses amebocyte lysates derived from blood of the horseshoe crab to quantitate endotoxin in protein, peptides, antibodies or nucleic acid samples. Amebocyte lysates are widely used as a simple and sensitive assay for the detection of endotoxin lipopolysaccharide (LPS), the membrane component of gram-negative bacteria. When endotoxin encounters the amebocyte lysate, a series of enzymatic reactions results in the activation of Factor C, Factor B and pro-clotting enzyme. The activated enzyme catalyzes the release of p-nitroaniline (pNA) from the colorless chromogenic substrate, Ac-Ile-Glu-Ala-Arg-pNA, producing a yellow color. After stopping the reaction, the released pNA is photometrically measured at 405 nm. The correlation between absorbance and endotoxin concentration is linear in the 0.1-1.0 EU/mL and in 0.01-0.1 EU/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

Note: Thorough cleanliness in labware, raw materials, and in lab technique is required to accurately detect levels of endotoxin in a given sample.

- 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.
- Maintaining the correct temperature is critical for reproducibility. Use a proper heating block at 37±1°C. Cabinet-style incubators are not recommended 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 recommended for making standard stock solutions.
- Each lysate lot is tested for functionality using the United States Reference Standard EC-6. The assay lot is then matched to a lot of our Endotoxin Standard (ES) by testing in parallel with the Reference Standard Endotoxin (RSE). The RSE/ES correlation assay determines the potency of each ES lot when used with each matching lysate lot.

Materials required but not supplied

- Disposable endotoxin-free glass tubes or pyrogen-free 1.5 mL microcentrifuge tubes
- Disposable endotoxin-free pipette tips
- Disposable endotoxin-free 96-well microplates or plate strips
- Stable temperature plate heater (37±1°C)
- Pipettor
- Repetitive pipettor (optional) or multichannel pipettor
- Pyrogen-free reservoir
- Microplate reader
- 25% acetic acid (stop solution)

Prepare materials

Note: Equilibrate all reagents to room temperature before use.

Prepare Endotoxin Standard Stock Solutions

1. Each *E. coli* Endotoxin Standard vial contains 10-50 EU of lyophilized endotoxin; the actual potency is printed on the label. Reconstitute with room temperature Endotoxin-Free Water by adding 1/10 mL of the EU amount indicated on the vial to make Endotoxin Standard (ES) Solution at 10 EU/mL (e.g., a vial with potency of 15 EU, when reconstituted with 1.5 mL of Endotoxin-Free Water (EFW), will yield a concentration of 10 EU/mL).
2. Vortex the solution vigorously for 15 minutes (recommended <1500 rpm).

   Note: 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 because the endotoxin adheres to the sides of the glass vial.
3. Prepare High Standards (0.1-1.0 EU/mL) (Table 1) or Low Standards (0.01-0.1 EU/mL) (Table 2) from the Endotoxin Standard Solution (10 EU/mL) using the dilutions and procedures in Tables 1 and 2.

Table 1 High Standards (0.1-1.0 EU/mL)

Vial	Volume of Endotoxin Standard Solution (mL)	Volume of Standard 1 (mL)	Endotoxin-Free Water (mL)	Final Endotoxin Concentration (EU/mL)	Vortex Time (min)
Standard 1	0.20	—	1.80	1.00	2
Standard 2	—	1.00	1.00	0.50	1
Standard 3	—	0.50	1.50	0.25	1
Standard 4	—	0.20	1.80	0.10	1
Blank	—	—	0.50	0	—

Table 2 Low Standards (0.01-0.1 EU/mL)

Vial	Volume of Endotoxin Standard Solution (mL)	Volume of Stock (mL)	Volume of Standard 1 (mL)	Endotoxin-Free Water (mL)	Final Endotoxin Concentration (EU/mL)	Vortex Time (min)
Stock	0.20	—	—	1.80	1.00	2
Standard 1	—	0.20	—	1.80	0.100	2
Standard 2	—	—	1.00	1.00	0.050	1
Standard 3	—	—	0.50	1.50	0.025	1
Standard 4	—	—	0.20	1.80	0.010	1
Blank	—	—	—	0.50	0	—

Reconstitute Lyophilized Amebocyte Lysate

1. **Reconstitute Lyophilized Amebocyte Lysate immediately before use** with 1.7 mL of Endotoxin-Free Water (EFW) and swirl gently to dissolve the powder. If more than 1 vial is required, pool 2 or more vials before use. **Avoid foaming; do not vortex the solution**.

   Note: Make sure to recover all of the powder from the sides and the cap of the vial by gently inverting end-over-end. Extreme care must be taken not to touch the inside part of the cap to avoid contamination.

   Note: Reconstituted amebocyte lysate solution is stable for 1 week at -20°C or colder if frozen **immediately** after reconstitution. Upon thawing, the reconstituted lysate solution may be used only 1 time. Once thawed, gently swirl the reagent to mix before use.

Sample preparation

- 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 red blood cells, and the diluted sample may need to be heat-shocked (70°C for 15 minutes).
- To stop all bacteriological activity in test samples, store samples to be tested at 2-8°C for <24 hours or -20°C for >24 hours.

Chromogenic Substrate

Each vial contains 3.4 mg of lyophilized Chromogenic Substrate. Reconstitute the substrate by adding 3.4 mL of Endotoxin-Free Water.

Note: Reconstituted Chromogenic Substrate is stable for 4 weeks when stored at 2-8°C. **Pre-warm a sufficient substrate amount for the assay to 37°C for no more than 5-10 minutes prior to use.**

Assay procedure

Note: Equilibrate all reagents to room temperature before use. Ensure pipetting order and rate of reagent addition remain consistent from well-to-well and row-to-row throughout the procedure.

1. Prepare all reagents and standards as directed in previous section immediately before use.
2. Pre-equilibrate plate in a heating block at 37±1°C. **Throughout the assay procedure, maintain the plate at 37±1°C.**
3. Add 50 µL of Endotoxin Standard dilutions, blank, and samples per well.

   Note: It is recommended to run each sample and standards in triplicate, including triplicate of a blank (50 µL of Endotoxin-Free Water).
4. Keeping the plate at 37±1°C, add 50 µL of the reconstituted Amebocyte Lysate Reagent per well. Begin timing as the lysate is added to the first well.
5. Once the Amebocyte Lysate Reagent has been added to the plate wells, briefly remove from the plate heater and mix by gently tapping 10 times on the side of the plate, avoiding spilling. Return the plate to the plate heater and incubate at 37±1°C for the time **T1 indicated on the lysate vial**.

   Note: T1 High = Time 1 for High Standards and T1 Low = Time 1 for Low Standards.
6. Reconstitute the Chromogenic Substrate as described in Material Preparation with 3.4 mL of Endotoxin-Free Water. Mix gently by tilting and swirling the vial. Pre-warm to 37±1°C for 5 minutes before use.
7. After exactly time T1, add 100 µL per well of pre-warmed reconstituted Chromogenic Substrate Solution.
8. Once the substrate solution has been added into all plate wells, briefly remove from the plate heater and mix gently by tapping 10 times to facilitate mixing. Return to the plate heater at 37±1°C for **T2 = 6 minutes**.
9. At exactly T2 = 6 minutes, add 50 µL per well of Stop Solution (25% acetic acid).
10. Once the stop solution has been added to the plate wells, remove the plate from the plate heater and mix by gently tapping 10 times on the side of the plate.
11. Read the optical density (OD) at 405 nm immediately after assay completion. If the plate is read at a later time, keep covered to avoid evaporation.
12. Subtract the average absorbance of the blank replicates from the average absorbance of all individual standards and sample replicates to calculate mean Δ absorbance.
13. 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.

    Note: Do not include the blank OD in the calculation of the regression line.
14. Use the formulated standard curve (linear regression) to determine the endotoxin concentration of each sample.

Table 3 Example data and standard curve for Low Standard.

UE/mL	Avg. OD (405 nm)	Δ	Std. Dev.	%CV
0.1	0.5120	0.3937	0.0137	3
0.05	0.3129	0.1945	0.0015	1
0.025	0.1851	0.0668	0.0001	0
0.01	0.1353	0.0170	0.0003	2
0	0.1183	0	0.0024	—

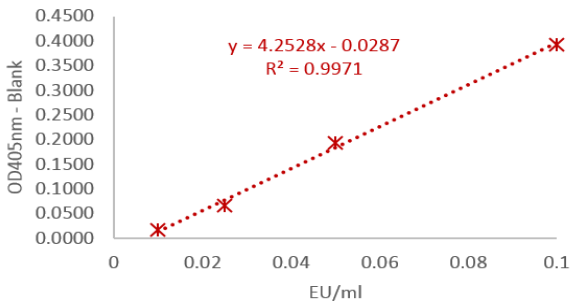
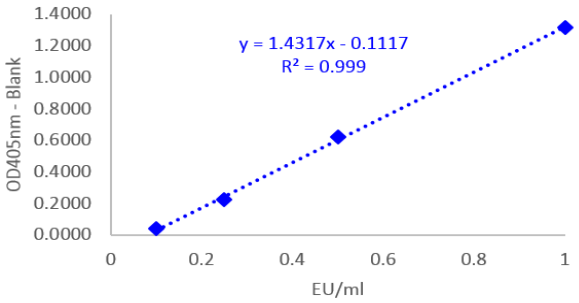


Table 4 Example data and standard curve for High Standard.

UE/mL	Avg. OD (405 nm)	Δ	Std. Dev.	%CV
1.0	1.4327	1.3162	0.0774	6
0.5	0.7382	0.6217	0.0158	3
0.25	0.3388	0.2223	0.0045	2
0.1	0.1581	0.0416	0.0018	4
0	0.1165	0	0.0033	—



Troubleshooting

Observation	Possible cause	Recommended action
Non-linear standard curve.	Endotoxin Standard Solution and dilutions were not mixed well.	Vortex the Endotoxin Standard Solution for 15 minutes before each use.
		Vortex all endotoxin standard dilutions for 1-2 minutes before each use.
		Vortex the endotoxin standard dilutions for 2 minutes if they were sitting for >10 minutes after preparation before adding into the plate wells.
	Pipetting order and rate of reagent addition were irregular.	Ensure pipetting order and rate of reagent addition remain consistent from well-to-well and row-to-row.
		Use a repetitive or multichannel 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 standard dilutions.	Materials (e.g., tips, vials, microplates) were contaminated.	Use endotoxin-free materials.
Higher absorbance in samples than standard curve.	Test sample endotoxin concentration is >1.0 EU/mL (for High Standard curve) or >0.1 EU/mL (for Low Standard curve).	Dilute the sample 5-fold in Endotoxin-Free Water. Re-test.
Samples turning yellow immediately after addition of the Stop Solution (25% acetic acid).	Samples contain substances that turns yellow in acidic environments (e.g., certain tissue culture media).	To determine if a sample's intrinsic color will alter the absorbance readings, construct a mock reaction tube by adding 50 µL of sample, 150 µL of Endotoxin-Free Water and 50 µL of Stop Solution with no incubation. Read the absorbance at 405 nm. 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.

Interfering substances

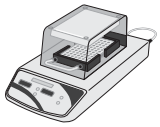
- The presence of interfering substances in test samples can cause product inhibition leading to false negatives. If unsure if your sample contains interfering substances, it is recommended to determine the potential product inhibition for each sample type undiluted or at an appropriate dilution (e.g., serum).
- To verify potential product inhibition, add a known amount of endotoxin to an aliquot or dilution of your test sample (e.g., 0.5 EU/mL). Assay the spiked sample and an unspiked sample to determine the respective endotoxin concentrations. The difference between the two calculated endotoxin values should equal the known concentration of the spike ±25%. See example below.
- Samples showing inhibition on the amebocyte lysate 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.

Table 5 Example with sample containing 20% glycerol.

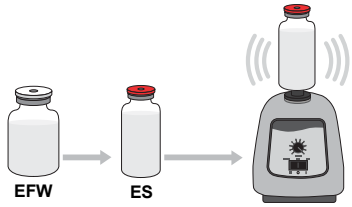
Sample Dilution	Observed Spiked[1] Sample Concentration (EU/mL)	Observed Unspiked Sample Concentration (EU/mL)	Δ	
Undiluted	0.103	0.099	0.004	Inhibitory
1:20	0.649	0.102	0.547	Non-inhibitory

[1] Spiked concentration should show a value of 0.50 EU/mL. The value of 0.103 is indicative of inhibition for sample containing 20% glycerol.

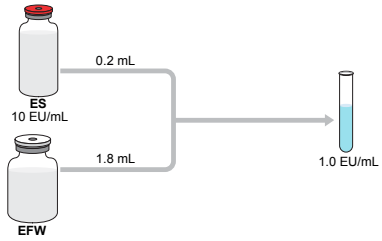
Workflow



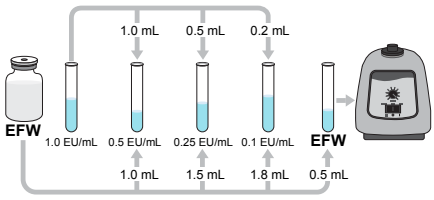
1. Pre-equilibrate the plate to 37±1°C for 10 minutes.



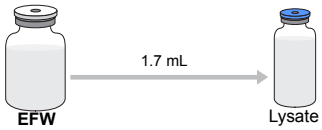
2. Reconstitute Endotoxin Standard (ES) to 10 EU/mL with Endotoxin-Free Water (EFW).
3. Mix vigorously for 15 minutes.



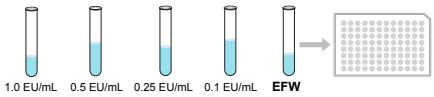
4. Prepare 1.0 EU/mL Stock from 10 EU/mL Endotoxin Standard Solution.
5. Vortex for 2 minutes.



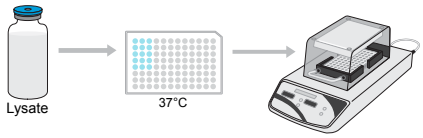
6. Using 1.0 EU/mL Stock, prepare a series of endotoxin dilutions.
7. Vortex for 1 minute.
8. Prepare samples according to information in the user guide.



9. Reconstitute the Amebocyte Lysate with 1.7 mL EFW.
10. Gently swirl and avoid foaming. Lysate reagent must be used within 5 minutes.

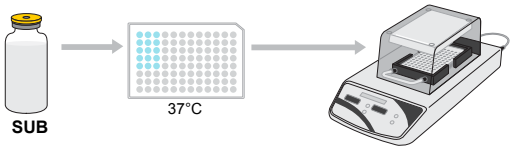


11. Keeping the plate at 37±1°C, add 50 µL of each endotoxin dilution, sample, and blank to the plate wells (triplicates).

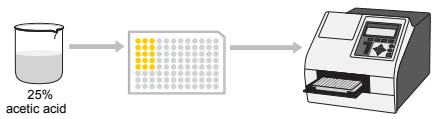


12. Keeping the plate at 37±1°C, add 50 µL of Amebocyte Lysate Reagent to the wells following the same order of addition as the endotoxin dilution and samples.
13. Mix well by gently tapping on the side of the plate.
14. Cover and incubate for time T1 (High or Low) as indicated on the Lysate label.

15. Reconstitute Chromogenic Substrate (SUB) with 3.4 mL of EFW.
16. Pre-warm the solution for 5 minutes at 37±1°C.



17. Keeping the plate at 37±1°C, add 100 µL of Chromogenic Substrate Solution to each well following the same order of addition as previous steps.
18. Mix well by gently tapping on the side of the plate.
19. Incubate for 6 minutes at 37±1°C.



20. Add 50 µL of 25% acetic acid to each well to stop the reaction.
21. Mix well by gently tapping on the side of the plate.
22. Read the absorbance at 405 nm.

Related products

Product	Cat. no.
Pierce™ High Capacity Endotoxin Removal Resin, 10 mL	88270
Pierce™ High Capacity Endotoxin Removal Resin, 100 mL	88271
Pierce™ High Capacity Endotoxin Removal Resin, 250 mL	88272
Pierce™ High Capacity Endotoxin Removal Spin Columns, 0.25 mL	88273
Pierce™ High Capacity Endotoxin Removal Spin Columns, 0.50 mL	88274
Pierce™ High Capacity Endotoxin Removal Spin Columns, 1 mL	88276
Detoxi-Gel™ Endotoxin Removing Gel	20339
Detoxi-Gel™ Endotoxin Removing Columns	20344
Pierce™ Rapid Gold BCA Protein Assay Kit	A53225

[1]Roslansky, P.F. and Novitsky, T.J. (1991). Sensitivity of Limulus amebocyte lysate (LAL) to LAL-reactive glucans. *J Clin Microbiol* **54** (5). Jcm.asm.org/content/29/11/2477.short

Limited product warranty

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