Endotoxin Gel-Clot Assay Kits

Catalog Numbers: GCT003 (16 tests)
GCT006 (16 tests)
GCT125 (16 tests)
GCT025 (16 tests)

PRODUCT DESCRIPTION

The R&D Systems Gel-Clot Endotoxin Assay Kit provides a reliable method for the visual detection of Gram-negative bacterial endotoxins (lipopolysaccharides) using the gel-clot formation technique. It utilizes Lyophilized Amebocyte Lysate (AL) Reagent derived from the circulating blood cells of the Asian horseshoe crab *Tachypleus amebocyte*.

When endotoxin is present, the serine protease proenzymes in the lysate are activated, converting coagulogen into coagulin and resulting in a gel-clot formation. If the endotoxin concentration meets or exceeds the assay's specified sensitivity (EU/mL), the gel remains intact after a 180° inversion of the reaction tube, indicating a positive result. Absence of gel-clot formation indicates that the endotoxin concentration is below the assays sensitivity threshold, signifying a negative result.

INTENDED USE

R&D Systems Gel-Clot Endotoxin Assay Kits are intended for *in vitro* detection of gram-negative bacterial endotoxins and are not for diagnostic use.

PRINCIPLE OF TEST

The gel-clot endotoxin assay involves mixing the AL Reagent with a test sample and incubating the mixture undisturbed at 37 °C for 60 minutes. The formation of a firm gel that remains intact when the tube is inverted indicates a positive result (endotoxin level is at or above the assay's sensitivity). In contrast, the absence of an intact gel signifies a negative result (endotoxin level is below the assay's sensitivity).

OTHER SUPPLIES REQUIRED

- Pyrogen-free pipettes, 0.2 mL, 1 mL, 5 mL, or automatic pipettors with pyrogen-free tips
- Vortex Mixer
- Heating block or non-circulating hot water bath (37 ± 1 °C)
- Timer
- Test tube rack

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MATERIALS PROVIDED & STORAGE CONDITIONS

R&D Systems™ Endotoxin Gel-Clot assays are offered in four sensitivity levels: 0.03, 0.06, 0.125, and 0.25 EU/mL.

Each kit provides components for 16 tests.

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

COMPONENT	CAP COLOR	QUANTITY PER TEST KIT	DESCRIPTION	STORAGE OF MATERIAL
Lyophilized Amebocyte Lysate (AL)	Blue	1 vial (1.7 mL/vial)	Produced from *Tachypleus tridentatus* amebocyte lysate. Reconstitute with Endotoxin-free water as specified on the vial label. The labeled sensitivity (λ) indicates the minimum Reference Standard Endotoxin concentration required to form a firm gel clot under standard test conditions. Sensitivity (EU/mL) is printed on the vial label.	Store at 2-8 °C. Avoid temperatures >20 °C and prolonged light exposure. Use reconstituted lysate within 10 minutes. If frozen immediately at ≤ -20 °C, stable for up to 28 days. **Do not refreeze**.
Endotoxin Control Standard	Red	1 vial	Prepared from *E. coli* strain 0111:B4. Each lyophilized vial contains 5-199 EU endotoxin. Potency is provided on the Certificate of Analysis (CoA). Reconstitute with Endotoxin-free Water using the volume stated in the CoA. Mix vigorously for 5 minutes on a vortex mixer.	Store at 2-8 °C. Reconstituted Endotoxin Control Standard is stable for 1 week at 2-8 °C. **Do not freeze.**
Endotoxin-free Water	Blue	1 vial (50 mL/vial)	Certified to contain < 0.005 EU/mL endotoxin. Used for reconstitution of Endotoxin Control Standard, dilution of endotoxin standards and samples, and as a negative control (blank).	Store at 2-30 °C.
Endotoxin-Free Test Tubes	NA	4 packs (5 tubes/pack)	Vacuum-sealed packs, dimension 10 x 75 mm.	Store at room temperature.
Endotoxin-Free Dilution Tubes	NA	2 packs (5 tubes/pack)	Vacuum-sealed packs, dimension 12 x 75 mm.	

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 an 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 microbial growth. 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 warm to room temperature before use. All solutions can be prepared at room temperature. Prepare Endotoxin Standards by reconstituting Endotoxin Control Standards immediately before use.

1. Reconstitute Endotoxin Control Standard using the Endotoxin-free Water volume listed in the kit's Certificate of Analysis to prepare a 50 EU/mL stock solution with the specified potency.
2. Endotoxin Standard Preparation (2λ, λ, 0.5λ, and 0.25λ)

CATALOG #	KIT SENSITIVITY	CONCENTRATION	INSTRUCTIONS
GCT003	-	5 EU/mL	Mix 0.2 mL of 50 EU/mL Endotoxin Stock Solution with 1.8 mL Endotoxin-free Water. Vortex for 1 minute.
	-	0.5 EU/mL	Mix 0.2 mL of 5 EU/mL endotoxin solution with 1.8 mL Endotoxin-free Water. Vortex for 1 minute.
	2λ	0.06 EU/mL	Mix 0.24 mL of 0.5 EU/mL endotoxin solution with 1.76 mL Endotoxin-free Water. Vortex for 1 minute.
	λ	0.03 EU/mL	Mix 1.0 mL of 0.06 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water. Vortex for 1 minute.
	0.5λ	0.015 EU/mL	Mix 1.0 mL of 0.03 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water. Vortex for 1 minute.
	0.25λ	0.0075 EU/mL	Mix 1.0 mL of 0.015 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water. Vortex for 1 minute.
	Blank	Blank	Use 0.5 mL of Endotoxin-free Water.
GCT006	-	5 EU/mL	Mix 0.2 mL of 50 EU/mL Endotoxin Stock Solution with 1.8 mL Endotoxin-free Water. Vortex for 1 minute.
	-	0.5 EU/mL	Mix 0.2 mL of 5 EU/mL endotoxin solution with 1.8 mL Endotoxin-free Water. Vortex for 1 minute.
	2λ	0.12 EU/mL	Mix 0.48 mL of 0.5 EU/mL endotoxin solution with 1.52 mL Endotoxin-free Water. Vortex for 1 minute.
	λ	0.06 EU/mL	Mix 1.0 mL of 0.12 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water. Vortex for 1 minute.
	0.5λ	0.03 EU/mL	Mix 1.0 mL of 0.06 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water. Vortex for 1 minute.
	0.25λ	0.015 EU/mL	Mix 1.0 mL of 0.03 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water. Vortex for 1 minute.
	Blank	Blank	Use 0.5 mL of Endotoxin-free Water.
GCT125	-	5 EU/mL	Mix 0.2 mL of 50 EU/mL Endotoxin Stock Solution with 1.8 mL Endotoxin-free Water. Vortex for 1 minute.
	-	0.5 EU/mL	Mix 0.2 mL of 5 EU/mL endotoxin solution with 1.8 mL Endotoxin-free Water. Vortex for 1 minute.
	2λ	0.25 EU/mL	Mix 1.0 mL of 0.5 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water. Vortex for 1 minute.
	λ	0.125 EU/mL	Mix 1.0 mL of 0.25 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water. Vortex for 1 minute.
	0.5λ	0.0625 EU/mL	Mix 1.0 mL of 0.125 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water. Vortex for 1 minute.
	0.25λ	0.031 EU/mL	Mix 1.0 mL of 0.0625 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water. Vortex for 1 minute.
	Blank	Blank	Use 0.5 mL of Endotoxin-free Water.
GCT025	-	5 EU/mL	Mix 0.2 mL of 50 EU/mL Endotoxin Stock Solution with 1.8 mL Endotoxin-free Water. Vortex for 1 minute.
	2λ	0.5 EU/mL	Mix 0.2 mL of 5 EU/mL endotoxin solution with 1.8 mL Endotoxin-free Water. Vortex for 1 minute.
	λ	0.25 EU/mL	Mix 1.0 mL of 0.5 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water. Vortex for 1 minute.
	0.5λ	0.125 EU/mL	Mix 1.0 mL of 0.25 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water. Vortex for 1 minute.
	0.25λ	0.0625 EU/mL	Mix 1.0 mL of 0.125 EU/mL endotoxin solution with 1.0 mL Endotoxin-free Water. Vortex for 1 minute.
	Blank	Blank	Use 0.5 mL of Endotoxin-free Water.

3. Discard the remaining endotoxin dilutions. Prepare a fresh endotoxin dilution series for every new experiment. Vortex the endotoxin solution for at least 1 minute immediately prior to each use.

REAGENT PREPARATION *CONTINUED*

Preparation of AL Reagent:

1. Reconstitute the lyophilized Amebocyte Lysate (AL) immediately before use.
2. Gently tap the vial to ensure all reagent powder settles at the bottom.
3. Add the labeled volume of Endotoxin-free Water to the vial.
4. Mix gently by tilting and swirling until the contents are fully dissolved.
   Note: *Do not vortex or shake, as this may cause foaming and denaturation of the reagent.*
5. Use the reconstituted lysate within 10 minutes.
6. If immediate use is not possible, freeze the reconstituted reagent at -20 °C or below immediately after reconstitution. Under these conditions, the reagent is stable for up to 28 days.
   Note: *The lysate should be frozen and thawed only once.*

PREPARATION OF CONTROLS

Positive Control: The positive control is an endotoxin solution prepared at a concentration of 2λ. In certain cases, it may be used in place of a full series of endotoxin standards to verify lysate reactivity.

Positive Product Control: Positive product controls consist of the test sample spiked with endotoxin at a concentration of 2λ. These controls are used to assess product interference or inhibition in the assay (see Product Inhibition section for details).

Negative Control: Use Endotoxin-free water as the negative control to confirm the absence of endotoxin contamination in reagents or materials.

TEST PROCEDURE

1. Add 0.1 mL Endotoxin-free water (negative control), endotoxin standards, or test sample into each endotoxin-free 10 x 75 mm glass test tube.
2. Add 0.1 mL of Lyophilized AL Reagent into each tube.
3. Mix gently but thoroughly. Failure to mix adequately is a common cause of unsatisfactory tests.
4. Incubate the mixture at 37 °C for 60 minutes, avoiding vibration during incubation. If large numbers of samples are tested in parallel, the tests should be batched and started at intervals. **Read the results within 5 minutes after incubation completed.**

RESULT READING

Remove and read reaction tubes one by one. Invert the tube in one smooth motion. A positive result is indicated by the formation of a gel that does not collapse when the tube is inverted. A negative result is characterized by the absence of a solid clot after inversion. Increase in turbidity or viscosity is considered negative result.

RESULTS AND INTERPRETATION

The results for all negative controls should be negative; positive results suggest the contamination of Lyophilized Amebocyte Lysate, Endotoxin-free Water, or glassware. Positive controls should yield positive results; a negative result may indicate loss of enzyme activity in the Lyophilized Amebocyte Lysate, reduced potency of Endotoxin Control Standard, or errors in endotoxin dilution. The results for positive product control should be positive. If the result for positive product control is negative whereas that for positive control is positive, the presence of interference in the test sample is hinted. See Product Inhibition section.

DETERMINATION OF THE MAXIMUM VALID DILUTION

The Maximum Valid Dilution (MVD) is the maximum allowable dilution of a substance to be examined at which the endotoxin limit can be determined. Determine the MVD using the following formula:

$$MVD = \frac{L \times C}{\lambda}$$

L refers to the endotoxin limit of the substance to be examined. L is specified in units such as EU/mL, EU/mg, or EU/Unit.

L = K/M, where K refers to the maximum allowable dose of endotoxin per kilogram body weight per hour.

M refers to the maximum allowable dose of substance per kilogram body weight per hour.

C refers to the concentration of the substance to be examined. It is expressed in mg/mL when the endotoxin limit is given by mass (EU/mg) and in Units/mL when the limit is based on biological activity (EU/Unit).

λ = The labeled Lyophilized Amebocyte Lysate sensitivity.

CONFIRMATION OF LABELED LYOPHILIZED AMEBOCYTE LYSATE SENSITIVITY (λ)

Run gel clot assay in 4 replicates with Lyophilized Amebocyte Lysate to be tested and endotoxin standard solutions at concentrations of 2λ, λ, 0.5λ, and 0.25λ. The test is valid only when results for all replicates of 2λ are positive, results for all replicates of 0.25λ and negative controls are negative. The labeled sensitivity is confirmed if the observed sensitivity is between 0.5λ and 2λ.

The observed sensitivity is equal to the Geometric Mean (G) of the endpoint (e).

$$G = (e1 \times e2 \times \ldots \times ef)^{1/f} = \lg^{-1}(\Sigma(\lg e)/f)$$

The endpoint (e) is the lowest endotoxin concentration with positive result in series, f is the number of replicate test tubes.

This is an example of the confirmation test for a sample with labeled Lyophilized Amebocyte Lysate sensitivity (λ) of 0.25 EU/mL.

Replicates f	Endotoxin Concentration (EU/mL)				Negative Control	Endpoint (EU/mL) e	Log10 e	Mean of Log10 e	G (EU/mL)
	2λ	λ	0.5λ	0.25λ					
	0.5	0.25	0.125	0.0625					
1	+	+	-	-	-	0.25	-0.602	-0.677	0.21
2	+	+	+	-	-	0.125	-0.903		
3	+	+	-	-		0.25	-0.602		
4	+	+	-	-		0.25	-0.602		

The observed sensitivity 0.21 EU/mL is between 0.125 and 0.5 EU/mL, therefore the labeled sensitivity 0.25 EU/mL is confirmed.

GEL CLOT SEMI-QUANTITATIVE ASSAY

Quantify the endotoxin concentration by finding the endpoint in a series of sample dilutions. In the following example, the sample is diluted with Endotoxin-free Water. Lyophilized Amebocyte Lysate sensitivity is 0.25 EU/mL.

Replicates	Endotoxin Concentration (EU/mL)						Negative Control	Endpoint Dilution	Log10 e	Mean	$Log10^{-1}$
f	1:2	1:4	1:8	1:16	1:32	1:64					
1	+	+	+	-	-	-	-	1:8 (0.125)	-0.903	-1.054	0.08 (1:11.3)
2	+	+	+	+	-	-	-	1:16 (0.0625)	-1.204		

Endotoxin Concentration = Lyophilized Amebocyte Lysate sensitivity x endpoint dilution
= 0.25 EU/mL x 11.3
= 2.83 EU/mL

GEL CLOT LIMIT TEST

Perform the test when a specification sets endotoxin limits. Run the diluted sample with positive product controls, positive controls, and negative controls using the MVD as the dilution factor. If the test is valid (see Result and Interpretation section), record the sample results.

Negative results for all sample replicates indicate that the endotoxin concentration is below the limit and the sample passes the gel clot test; positive results for all replicates indicate that the concentration exceeds the limit and the sample fails; if results are mixed, the test should be repeated with four replicates, and the sample is considered to pass only if all four replicates are negative.

PRODUCT INHIBITION/ENHANCEMENT

Product inhibition test is frequently employed to evaluate the existence of interference in the sample. Prepare a series of two-fold dilutions of endotoxin in both Endotoxin-free Water and sample matrix, run assay with two parallel series and calculate the geometric mean endpoint for each series.

If the geometric mean endpoint of endotoxin in sample matrix is within the range of 0.5λ-2λ, the sample is considered free of product inhibition. Otherwise, the existence of interference in the sample is suggested.

Product inhibition is usually concentration dependent, and could be reduced by dilution with Endotoxin-free water. The dilution should not exceed the MVD. The application of more sensitive Lyophilized Amebocyte Lysate allows greater sample dilution, which may improve the elimination of interference.

REFERENCES

- U.S. Department of Health and Human Services, Food and Drug Administration. Guideline on Validation of the Limulus Amebocyte Lysate (LAL) Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices. Public Health Service; December 1987.
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