

Jan 15, 2026

Determination of LD₅₀ and LD₉₀ for Bacterial Pathogens in *Galleria mellonella*

DOI

<https://dx.doi.org/10.17504/protocols.io.5qpvo13o9g4o/v1>

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Galleria protocols



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Protocol Citation: Juan Jose Quispe Haro 2026. Determination of LD₅₀ and LD₉₀ for Bacterial Pathogens in *Galleria mellonella*. protocols.io <https://dx.doi.org/10.17504/protocols.io.5qpvo13o9g4o/v1>

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Protocol status: Working

We use this protocol and it's working

Created: January 15, 2026

Last Modified: March 04, 2026

Protocol Integer ID: 238688

Keywords: *Galleria mellonella*, LD50 determination, virulence assay, bacterial pathogenesis, infection model, Probit analysis, Spearman-Kärber method, in vivo screening, Uropathogenic *Escherichia coli*, UPEC, Id90 for bacterial pathogen, meier survival curves for each bacterial dose, *Galleria mellonella* larvae, standardized infection doses for later experiment, bacterial dose, *Galleria mellonella* this protocol, direct comparison of pathogen, standardized infection dose, bacterial pathogen, determination of Id50, *Galleria mellonella*, uropathogenic *Escherichia coli*, infection model, pathogen, adaptable to other bacterial species, other bacterial species, LD₅₀, Id90, Id50, greater wax moth, meier survival curve, lethal dose, Id₉₀

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Abstract

This protocol describes a method for determining the median and 90% lethal doses (LD₅₀ and LD₉₀) of bacterial pathogens using the *Galleria mellonella* larvae (greater wax moth) infection model. The protocol is optimized for uropathogenic *Escherichia coli* (UPEC) but is readily adaptable to other bacterial species. Expected results include Kaplan-Meier survival curves for each bacterial dose and precise LD₅₀/LD₉₀ values with confidence intervals, calculated via Probit or Spearman-Kärber analysis. These metrics allow for direct comparison of pathogen strains and establish standardized infection doses for later experiments.

Guidelines

Calculation of the LD₅₀ and LD₉₀ can also be done outside of Prism in software like MS Excel. Use this formula if you prefer a manual calculation or lack software. It requires doses that yield 0% and 100% mortality at the extremes.

$$LD_{50} = \log_{10}(x_k) + (\log_{10}(d) * (0.5 - \sum p_i))$$

Where x_k is the lowest dose that gives 100% mortality (or the last overall), d is the Log-interval between doses (10 for 10-fold dilutions), Sum p_i is the sum of the proportions of mortality for all doses up to, but NOT including x_k.

For LD₉₀, replace 0.5 for 0.9 in the equation.

Always report:

The timepoint used for the LD calculation.

The method used (e.g., Probit analysis via GraphPad Prism).

The LD value with its 95% Confidence Interval (e.g., LD₅₀ = 4.9 × 10⁶ CFU/larva [95% CI: 2.1×10⁶ - 1.1×10⁷]).

The sample size (n) per group.

Materials

Sterile PBS

2 ml Eppendorf tubes

Crushed ice tray

Refrigerated ultracentrifuge

Hamilton syringes with a 30G needles

Safety warnings

- ⚠ Ensure all equipment and reagents are sterile to prevent contamination.
Take special care when manipulating needles and syringes to avoid accidental punctures.

Ethics statement

Ensure to follow all safety and ethical guidelines when handling live larvae and biological materials.



The day before

16h

- 1 Inoculate 5 ml of appropriate sterile media with a single colony of the target *E. coli* strain.
- 2 Incubate overnight at 37°C with vigorous shaking (200 rpm).

16h



Larval Preparation

- 3 Select healthy, 7th-instar larvae (>300 mg), with no signs of melanization or discoloration.
- 4 Randomly allocate 16 larvae per treatment group.
- 5 Optional: Place larvae on ice for 5-10 minutes to immobilize them.
- 6 Clean the injection site (last left proleg) with 70% ethanol and allow to air dry.



Bacterial Suspension preparation

- 7 Transfer 2ml of the overnight culture to a 2 ml microcentrifuge tube.
- 8 Centrifuge at 600 x g for 10 minutes, at 4 °C.
- 9 Carefully discard the supernatant.
- 10 Resuspend the pellet in 1 ml of sterile, ice-cold PBS.
- 11 Measure the OD₆₀₀. Adjust the suspension with PBS to a final OD₆₀₀ = 1.25 $\approx$ 1 × 10⁹ CFU/ml.





- 12 Perform 10-fold serial dilutions in sterile PBS to create the following working stocks:
 - Group 1: 1×10^8 CFU/mL
 - Group 2: 1×10^7 CFU/mL
 - Group 3: 1×10^6 CFU/mL
 - Group 4: 1×10^5 CFU/mL
 - Group 5: 1×10^4 CFU/mL
 - Control: Sterile PBS only.
- 13 Keep all bacterial suspensions on ice until injection. 

Infection

- 14 In a clean environment, prepare one Hamilton syringe with a 30G needle for each experimental group.
- 15 Clean the syringes and needles by drawing up and down a solution of 70% ethanol several times, then allowing them to air dry. 
- 16 Using one syringe, draw up 10 μ L of the appropriate suspension and inject the 10 μ L of the suspension into the hemocoel via the last left proleg.
- 17 Place injected larvae in a Petri dish (max 10 larvae/dish).
- 18 Incubate dishes at 37°C under normal day/night cycle illumination.  

Survival monitoring

- 19 Check larvae at the following time points post-injection:
2, 4, 6, 8, 10, 24, 48, 72, 96, and 120 hours.
- 20 Record the number of deaths at each timepoint until 100% mortality is achieved.

Survival curves calculation

- 21 In GraphPad Prism, create a new "Survival Table".



- 22 Input the death datapoints as 1 in the respective columns. All larvae that survived until the end should be input as 0 in the final timepoint.
- 23 Generate a Kaplan-Meier survival curve. Use the Log-rank (Mantel-Cox) test to statistically compare curves between groups.

LD curves calculation

- 24 Select a single, relevant endpoint (e.g., 24h or 48h), when mortality has plateaued for mid-range doses.
- 25 In GraphPad Prism, create a new "XY Table".
- 26 Input the $\text{Log}_{10}(\text{Dose})$ (e.g. 6 if the concentration was 10^6 cells/ml) in the X column, and "% Mortality" in the Y column.
- 27 Go to Analysis > XY analyses > Nonlinear regression (curve fit). Choose: Dose-response - Stimulation → Probit model. Prism will output LD_{50} and LD_{90} with 95% Confidence Intervals.