

Gentamicin Protection Assay to Determine Bacterial Survival within Macrophages

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[Abstract] Macrophages are key cells involved in orchestrating host defense against infections. Here, we describe the protocol for a bacterial killing assay in macrophages that can be adapted to any bacterial pathogen. Using this assay, we analyzed the survival of wild-type and mutant strains of *Escherichia coli* (*E. coli*) within RAW 264.7 cells, a widely used macrophage cell line. Bacterial mutants defective in intracellular survival within macrophages can be delineated using this assay.

Materials and Reagents

1. RAW 264.7 cells (ATCC, TIB-71)
2. *E. coli* CFT073 or other strains of bacteria
3. LB broth (Beckton Dickinson, catalog number: 244610)
4. Agar (Beckton Dickinson, catalog number: 214010)
5. RPMI1640 (Life Technologies, catalog number: 11875-093)
6. Gentamicin 50 mg/ml (Life Technologies, catalog number: 15750-102)
7. PBS (pH 7.4)
8. LB plates (see Recipes)
9. Certified, heat inactivated fetal bovine serum (FBS) (Life Technologies, catalog number: 10082139) (see Recipes)
10. 100x penicillin, streptomycin and glutamine (PSG) (Life Technologies, catalog number: 10378-016) (see Recipes)
11. Saponin (Sigma-Aldrich, catalog number: 8047-15-2) (see Recipes)

Equipment

1. 24 well plates (Corning, Costar®, catalog number: 3524)
2. 0.22 micron filter (Millipore, catalog number: SCGP00525)
3. 37 °C, 5% CO₂ incubator
4. Sorvall Legend RT table top centrifuge

5. Shimadzu UV-1601PC Spectrophotometer

Procedure

1. Culture RAW 264.7 cells in RPMI1640 with 10% heat inactivated fetal bovine serum and 1x penicillin streptomycin and glutamine at 37 °C in a CO₂ incubator.
2. Count the cells using standard procedures and seed 2 x 10⁵ RAW cells/well, by adding 1 ml per well in a 24 well plate. Set up 2 plates; one for T0 and another for T2 and incubate in a CO₂ incubator.
3. Start overnight cultures of CFT073 in 3 ml of LB broth in a 10 ml polypropylene tube and incubate at 37 °C shaking at 200 rpm. Use appropriate culture media when using other bacterial strains.
4. Determine the multiplicity of infection (MOI = number of bacteria per RAW 264.7 cell) and alter the inocula preparation accordingly. For an MOI of 10, add 2 x 10⁶ cfu of *E. coli* CFT073 for a well containing 2 x 10⁵ RAW cells.
5. Pellet the bacterial cells from the 3 ml culture by centrifuging at 4,000 rpm for 10 min in a Sorvall centrifuge. Resuspend the pellet in 3 ml PBS.
6. Adjust the OD₆₀₀ of broth culture to 4 in PBS, by measuring the optical density of a 1:10 diluted culture in a spectrophotometer and calculating the optical density of undiluted culture.
7. Dilute 1:100 in PBS.
8. Dilute 1:10 in RPMI 1640 without antibiotics.
9. Wash RAW cells twice with PBS.
10. Add 1 ml of diluted culture to each well; three technical replicates per sample and three biological replicates of the entire experiment are recommended. Use the same set up for T0 and T2 plates.
11. Incubate at 37 °C, 5% CO₂ for 30 min.
12. Aspirate supernatant and wash 3 times with PBS.
13. Add 1 ml of RPMI 1640 with gentamicin (200 µg/ml) per well.
14. Incubate the T0 plate at 37 °C, 5% CO₂ for 15 min.
15. Incubate the T2 plate at 37 °C, 5% CO₂ for 2 h.
16. After 15 min or 2 h, remove the supernatants entirely by aspiration.
17. Lyse cells with 1 ml of filter sterilized 1% saponin in H₂O per well by incubating for 10 min at room temperature followed by vigorous pipetting. Determine the extent of lysis using a standard inverted microscope.
18. Plate undiluted and -2 dilution for T0 and T2 time points on LB plates.
19. % Killing = [(T0-T2)/T0]*100

Recipes

1. LB plates
Add 15 g of agar to 1 L of LB broth
2. FBS and PSG
Added at 10% and 1x final concentration, respectively, to RPMI1640
3. Saponin
Dissolved in autoclaved double distilled water and filter sterilized using a 0.22 μ M filter

Acknowledgments

This protocol was adapted from the publication: Subashchandrabose *et al.* (2013).

References

1. Subashchandrabose, S., Smith, S. N., Spurbeck, R. R., Kole, M. M. and Mobley, H. L. (2013). [Genome-wide detection of fitness genes in uropathogenic *Escherichia coli* during systemic infection](#). *PLoS Pathog* 9(12): e1003788.