BMDM Salmonella Replication

Rabia Khan

Abstract

Citation: Rabia Khan BMDM Salmonella Replication. **protocols.io**
dx.doi.org/10.17504/protocols.io.gcrbsv6
Published: 07 Nov 2016

Protocol

Step 1.
The day before:. Inoculate 5 ml of TSB with 1 colony of Salmonella Typhimurium. Incubate on the wheel at 37C overnight.

Step 2.
Collect BMDM using a cell scraper. Transfer the cells from one Petri dish to a 50-ml tube. Centrifuge 10 min at 1200 RPM. Remove and discard the supernatant. Re-suspend the cells using 3-ml syringe and needle (start with a 21 or 20 gauge needle and finish with 25 gauge in 2 ml RPMI + 10% FCS. Count BMDM using the Coulter Counter or a 1/5 dilution with Turks. (1.5ml Acetic acid, 48.5 ddH2O and Gentacin Violet – trace).Adjust with fresh cell culture medium the number of cells to a final concentration of 1x10^6 cells per ml. Aliquot 500 µL (5x10^5 cells) per well for a 24-well plate. Aliquot 3 mL (3x10^6 cells) per well for a 6-well plate. And 100ul for 96 well plate. Incubate o/n at 37C, 5% CO2. For LDH, all the samples are in triplicate.

Step 3.
The day of the experiment:Inoculate 50 ml of TSB with 2 ml of the overnight culture of Salmonella. Grow at 37C with agitation until the OD at 600nm reaches 0.9 (it takes between 90-120 min). Place the bacterial culture on ice and adjust the OD to 0.9 if needed.In the meantime, prepare all the media which need to be kept at 37CMedium in which to add bacteriaPBS for the washesGentamicin +RPMI+10%FCSTitron and PBS for lysis

Step 4.
Centrifuge two tubes of 1 ml (5 x 10^8 bact) of the bacterial culture and resuspend in 1 ml of saline or PBS or RPMI without FCS. Use Eppendorf tube and centrifuge in the cold room 3-4 min at 13000 RPM.

Step 5.
Verify that the BMDM are healthy under the microscope. Remove the cell culture medium and replace it with RPMI and 10% FCSPer well:6-well plate: 3 ml RPMI +10%FCS and 60ul bacteria24-well plate: 500 µL RPMI +FCS and 10ul bacteria96 well plate: 100ul RPMI+10%FCS and 2ul bacteria(10 µL corresponds approximately to 5x10^6 bacteria; MOI of 10 bacteria/cell).For a 6-well plate, prepare 20 ml of culture medium + 400 µl of washed bacteria.For a 24-well plate, prepare 15 ml of culture medium + 300 µl of washed bacteria.For a 96 well place, prepare 10ml of culture medium and 200ul of washed bacteria. But for LDH, you only need to prepare enough for the Salmonella wells, not for all 96 wells.

Step 6.
Centrifuge at 500g for 5 minutes at 25C

Step 7.
Incubate 45 min at 37C, 5% CO2.

Step 8.
During the incubation time, plate the infectious dose 10^-3 (100 µl), 10^-4 (100 µl) and 10^-5 (100 µl).

Step 9.
After the incubation period, remove and discard the supernatant. Wash the cells twice with PBS (preheated at 37C) (prepared before hand) using:500 µl for 24-well plate and

Step 10.
ml for 6-well plate.100ul for 96 well plate10. Add 500 µL (24-well plate) or 2 ml (6-well plate) RPMI 10% FCS without phenol for LDH (preheated at 37C) containing 100 µg/ml gentamicin. Add an extra triplicate for medium.For a 24-well plate, prepare 15 ml of culture medium + 150 µl of gentamicin 10 mg/ml. For a 6-well plate, prepare 20 ml of culture medium + 200 µl of of gentamicin 10 mg/ml.For a 96 well plate, prepare 15ml of culture medium + 150ul of gentamicin at 10mg/mlThis corresponds to time 0 for LDH.Start timer for time points and 45 min prior to last time point for max rel.For all time points: spin plate at 250g for 4min*keep on ice*Take 50ul of supernatant for LDH into special LDH plates (can use multichanel) and keep on ice.Take 50ul for ELISA cytokine expressionLyse cells with 100ul of PBS-1% Titron X-100 (prepared in advance)Tranfer 100ul to 2ml push cap tubes for plating.Dilute 50ul in 450ul for 10^-1 and 10^-2#CFUs/50µl x 100 for LDH x dilution = CFUs per wellContinue on for Non LDH experiment:

Step 11.
Incubate 1 hour at 37C, 5% CO2

Step 12.
After the incubation period, remove and discard the supernatant. Wash the cells twice with PBS (preheated at 37C) using500 µl for 24-well plate

Step 13.
ml for 6-well plate.Add 500 µL (24-well plate) or 2 ml (6-well plate) RPMI 10% FCS (preheated at 37C) containing 10 µg/ml gentamicinFor a 24-well plate, prepare 15 ml of culture medium + 15 µl of gentamicin10 mg/ml.For a 6-well plate, prepare 20 ml of culture medium + 20 µl of of gentamicin 10 mg/ml.Pursue the incubation (1, 2, and 4 h).Time 0 corresponds to the time just after adding the RPMI containing 10µg/ml gentamicin.At each time point, collect the supernatants for future cytokine ELISA assays and freeze them at -80C. Lyse the cells with 500 µL PBS- 1%Triton X-100 and transfer the lysate to 4-ml push cap tube.

Step 14.
Dilute the lysate (10-1 to 10-3). Spread 100 µL of 10-2 and 10-3 dilutions on TSB Petri dishes and incubate overnight.The day after:

Step 15.
Count the CFUs taking into account the volume plated and the dilution.#CFUs/100µl x 500 x dilution = CFUs per well

Step 16.