Xenophagy analysis (Autophagy against *Salmonella*)

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METHOD

Preparation of *Salmonella*

1. Inoculate *Salmonella* (from a single colony) into 3 ml of LB medium in a 15 ml tube with loose cap.
2. Incubate overnight at 37°C in an incubator with shaking (~16 h).
3. Inoculate 100 ul of the preculture into 3.3 ml of LB medium in 15 ml tube with loose cap (1:33).
4. Incubate with statically for 3 hours at 37°C in an incubator.
5. Harvest the *Salmonella* by centrifugation at 10,000 x g for 2 min.
6. Remove the supernatant and add an equivalent volume of sterile PBS.
7. Harvest the *Salmonella* as in step 5.
8. Remove the supernatant and add an equivalent volume of DMEM.

NOTE DMEM does not contain any antibiotics.

9. Measure OD_{600} of the bacterial culture.

NOTE A bacterial culture containing 1.2×10^9 CFU/ml give the OD_{600} of 1.0.

Invasion of *Salmonella*

1. On the day of the *Salmonella* inoculation (as described above), seed cells in 12-well plates in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 2 mM L-glutamine, and maintain the cells overnight at 37°C with 5% CO_2.

NOTE As a xenophagy-negative control, we use *Atg5* or *Atg7* KO MEFs.

NOTE DMEM does not contain any antibiotics.

NOTE Cells should be actively growing in logarithmic phase at the time of infection

2. Inoculate cells with *Salmonella* by adding bacteria directly to the cell culture supernatant (MOI of 10-100).
3. Incubate for 2-10 min at 37°C.

NOTE Strict adherence to infection time.

4. Wash the cells twice with PBS.

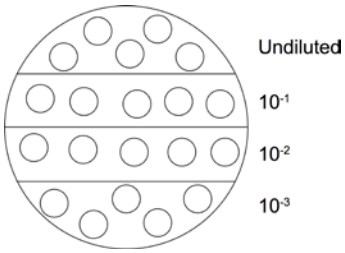
5. Add DMEM without antibiotics and incubate for 20 min.
6. Change medium containing 50 ug/ml gentamicin.
7. Incubate for 40 min at 37°C.
8. Change medium containing 5 ug/ml gentamicin.

Colony formation assay

1. Inoculate cells with *Salmonella* as described above.
2. Incubate the cells for ~8 hours at 37°C. (infection time should be optimized for each cell type but 1 h after infection should be always included as the normalization time point (see below))
3. Wash the cells with PBS twice.
4. Add 1 ml of lysis buffer and collect the lysate into 1.5 ml tube.
5. Dilute the lysate with PBS (1:10, 1:100, and 1:1000).
6. Spot 10 ul of each dilution on LB plate with or without appropriate antibiotics.

NOTE More than three spots are required on each dilution.

7. Incubate overnight at 37°C.
8. Count colonies on one of the appropriate dilution.



NOTE If total 20 colonies in the 5 spots of undiluted, then total cfu/well = 20/5 x 100 = 4.0 x 10^2.

NOTE If total 20 colonies in the 5 spots of 1:1000, then total cfu/well = 20/5 x 100 x 1000 = 4.0 x 10^5.

NOTE The normalize point is set to 1 hour after infection.

MATERIALS

REAGENTS

• Mouse embryonic fibroblasts (MEF)
• *Salmonella* (*Salmonella enterica* serovar Typhimurium)
• Dulbecco's modified eagle's medium - high glucose (Sigma, D5796)
• Trypsin-EDTA (0.25%) (Life technologies, 25200-056)
• Fetal bovine serum (Equitech-Bio, Inc), heat inactivated (56 ˚C, 30 min)
• Penicillin-Streptomycin (5,000 U/mL) (Life technologies, 15070-063)
• MEM Non-Essential Amino Acid Solution, 100x (Life technologies, 11140-050)
• Sodium Pyruvate (100 mM) (Life technologies, 11360-070)

• L-glutamine (200 mM) (Life technologies, 25030-081)
• DPBS (Nissui Pharmaceutical, 08190)
• LB Broth (Life technologies, 12780-052)
• Agar (Nacalai tesque, 01028-85)
• Gentamycin (Life technologies, 15750-060)
• X-SAL agar medium (Nissui Pharmaceutical, 05140)

EQUIPMENT

• Costar® 6 Well Clear TC-Treated Multiple Well Plate (CORNING, #3516)
• Costar® 12 Well Clear TC-Treated Multiple Well Plate (CORNING, #3513)
• Liquid aspirator setup (ULVAC, DAP-15)
• CO_2 incubator (Panasonic, MCO-20AIC)
• 15 ml tube (Greiner bio-one, 188271)
• Thermostatic chamber (Fukushima Industries Corporation, FMU-2631)

REAGENT PREPARATION

PBS for cell culture

Dissolved 9.6 g of DPBS in 1000 ml of distilled water and autoclave (121 ˚C, 30 min). This reagent can be stored at room temperature.

Lysis Buffer

• 1% (v/v) TX-100
• 0.1% (v/v) SDS

Dissolved 10 ml of 10% TX-100 and 1 ml of 10% SDS in 100 ml of PBS. Autoclave at 121°C for 20 min. This reagent can be stored at room temperature.

X-SAL agar medium

Add 68.2 g of X-SAL agar medium in 1000 ml distilled water and heat the medium. After dissolved the powder completely, dispense 20 ml of the medium on a plate. This agar plate can be stored at 4°C.

TROUBLESHOOTING TIPS

1. Notes of pathogen handling

Pay close attention not to spreading of contamination.

When the bench is contaminated with bacteria, it should be sterilized immediately using ethanol for disinfection, povidone iodine, or sodium hypochlorite for appropriate period.

2. Poor growth efficiency of *Salmonella*.

   The growth of *Salmonella* is affected by temperature. Refrain from opening of the CO_2 incubator lid as much as possible. *Atg5* or *Atg7* KO MEFs can be used for a positive control of whether the experiment well done, because replication rate of *Salmonella* in the cells is high more than 2 times compared with wild-type cells.

3. Cannot be taken the reproducible results.

   Infection efficiency of *Salmonella* strongly affects the results. Strict adhere to the number of cells and infection time to the cells.