Infection of HIEC-6 cells with *Salmonella*

Day 1:

1) Streak strains from frozen glycerol stock onto BHI agar plates. Incubate at 37°C for 24 – 26 h.

2) Seed cells according to your experimental design, some of the most frequently used designs are included below for reference

Plate	Hours until confluent	Seeding Density
6-well plate	72 hr	2 x 10^5 cells in 2 mLs
24-well plate on glass coverslips	72 hr	1 x 10^5 cells in 1 mL

Day 2:

1) Inoculate single colonies with 5 mL volumes of LB 0.3 M NaCl pH 8 media in red-capped tubes. Grow stationary, at 37°C for 12 – 14 h.
   Note: the high salt, high pH media is used to induce expression of SPI-1 genes for enhanced invasion. Essentially, these bacteria are 'primed' for invasion.

Day 3:

1) Check tissue culture cells and confirm that they are between 60-70% confluent and are free of contaminants.

2) Prepare media and pre-heat at 37°C in a waterbath:
   For 100 ug/mL gentamicin treatment: 1 uL of 50mg/mL gentamicin stock per mL of media
   For 10 ug/mL gentamicin treatment: 0.1 uL of 50 mg/mL gentamicin stock per mL of media

3) Sub-culture 1:100 (so 50 uL into 5 mL) bacterial cultures. Target OD600 is 0.4-0.5 (this is ~4 hours for FSL S5-0395 and its various mutant strains) after sub-culturing, but you can use strains up to 4.25 hours post inoculation)

4) Calculate the desired concentration of bacteria for the inoculum
   a. *Note: at OD_{600} = 0.4-0.5 FSL S5-0395 is at 5 x 10^8 cfu/mL*

5) When bacterial cultures are at OD_{600} ~0.4, transfer 500 µL to a clean 1.5 mL Eppendorf tube. Add the calculated number of cells to their respective wells in the tissue culture plate.
   a. *Note: It is helpful to immerse the pipette tip (with the culture) into the tissue culture media in the well plate. This way you will avoid 'spraying' the Salmonella cells into the nearby wells.*

6) Return HIEC-6 cells to the 37°C incubator. Incubate infected cells for 1 hr.

7) After one hour remove media with a Pasteur pipette and, wash 3X with PBS.
   a. *Note: to perform the washes, first remove the spent media with a Pasteur pipette, then add 1 mL of pre-warmed PBS. Remove the PBS and repeat.*

8) Add media containing gentamicin 100 ug/mL and incubate for 1 hr.

9) Wash 3X with pre-warmed PBS as described above, add media containing 10 ug/mL gentamicin and incubate for 48 h and process the cells according to the desired experimental procedure (i.e., flow cytometry, immunofluorescence staining, etc.).