

Minimal inhibitory concentration (MIC) & minimal bacterial concentration (MBC) assays

Materials:

- LB (Sterile)
- MilliQ water (Sterile)
- Double LB (Sterile)
- LB agar plates
- 14 ml bacterial culture tubes with lids
- Falcon tubes 15 ml & 50 ml
- Pipette tips
- multichannel pipette
- EPOCH Plate Reader
- 96-well plate with lid (round bottom)
- Anti-Fog Solution (Recipe Below)
- Compounds for testing at desired concentration in water
- Bacterial strain *E.coli*-DH5 α (or other) (-80°C stock)
- Eppendorf Bio Photometer
- Plastic cuvettes 1 ml
- Reservoir

Work under sterile conditions

Methods:

Day 1 → Streak out (single-colony method) bacterial strain (*E. coli*) on LB Agar plate as shown below and allow to incubate overnight (ON) at 37°C.

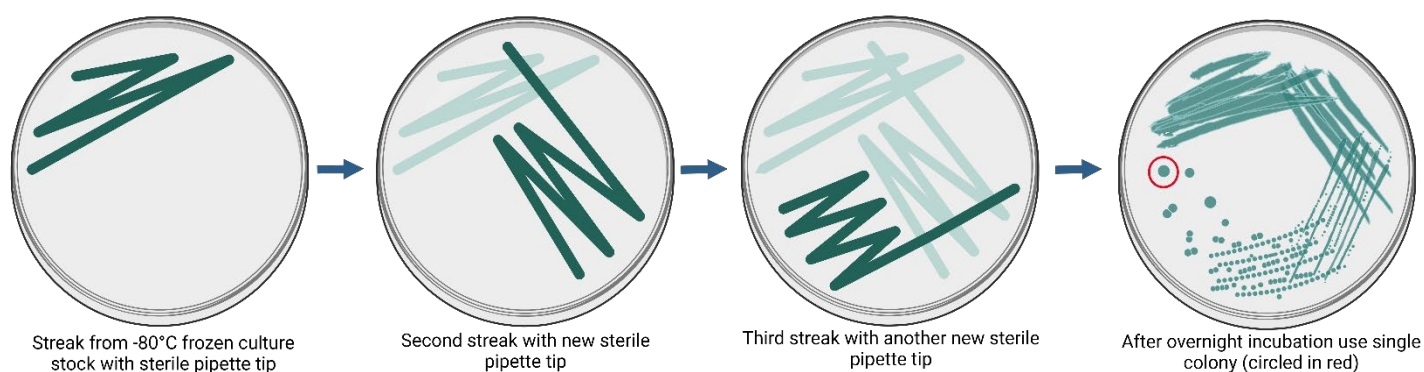


Figure 1: Single colony streak out method

Day 2 → Prepare bacterial culture by inoculating 5mL LB with single colony from plate. Prepare negative control of only LB (3mL). Grow shaking 180-250 rpm at 37°C and allow to grow 6-16 hrs.

Confirm that the negative control LB is still clear and sterile. In the clean bench, apply antifog solution (0.05% TritonX-100, 20% Ethanol in water) to the inside of 96 well lid, fully cover with solution and pour back the excess solution. Allow the lid to fully dry (or alternatively dry with kimwipes™).

Fill wells A2-12 with 100µL of sterile MilliQ water. To the first well (A1) add 200µL of compound solution. Perform a serial two-fold solution (take 100µL from the first well and mix it with the second well, then 100µL from the second well and mix it with the third), change pipette tips when going down in the concentration gradient. Throw away the last 100µL from the last well (A12) so that the same volume is in each of the wells. Perform same set up in row B (this will be later used as a blank).

Measure OD₆₀₀ of *E. coli* culture and $C_1V_1=C_2V_2$ formula make stock bacterial solution using double LB with a final bacterial concentration of 0.02 OD₆₀₀. Add 100µL of this stock to all of the wells in row A. Add 100µL of double LB to all of the wells in row B. Your final starting bacterial concentration will be 0.01 OD₆₀₀.

Repeat the previous steps for additional rows depending on how many compounds are being tested.

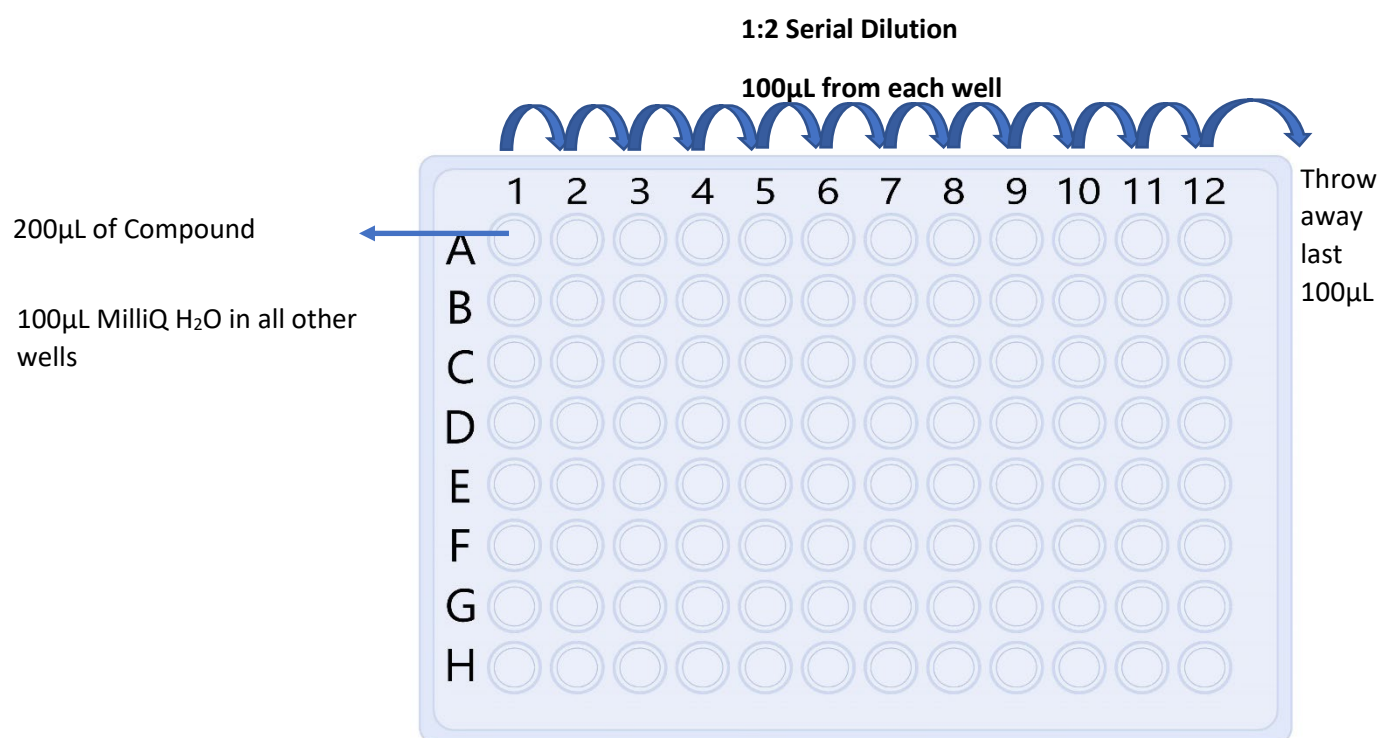


Figure 2: 96-Well Plate Set-Up of Serial Dilution

Cover plate and place in EPOCH 2 plate reader with continuous shaking (double orbital) at 37°C measuring at OD₆₀₀ in 15 min intervals for 20 hours. Alternatively, place in shaking incubator at 37°C and measure OD₆₀₀ after 20 hours of growth.

Day 3 → Export data into excel, save and analyze for growth curve analysis portion.

MIC is determined by visual examination: the lowest concentration at which no visible bacterial growth appears should be recorded as the MIC value. All tests should be repeated in three replicates each time (technical repeats), and then repeated again three times on different days/different single colony overnight cultures (biological repeats).

MBC is determined as the lowest concentration of the antimicrobial agent that killed 99.9% of the initial inoculum. 20 μ L of aliquots bacterial suspensions from the wells without viable cells, where bacterial growth was inhibited, are to be inoculated onto the surface of LB agar plates and incubated under anaerobic conditions for 48 h at 37°C with 5% CO₂. After calculating the colony-forming units (CFU), the MBC value can be determined. (See CFU protocol).

Day 4 → Using colony counter and marker, count the colonies in each drop and write the numbers down and the corresponding dilution. Record the numbers in lab notebook. Analyze values in excel applying the following formula for the relationship between number of colonies, volume plated (20 μ L) and dilution factor:

$$\frac{CFU}{mL} = \frac{(number\ of\ colonies\ \times\ dilution)}{volume\ plated}$$

Then graph the resulting CFU/mL as bar graphs using standard deviation values as error bars values.



Figure 3: LB Agar plate after colonies have formed and counted using marker