Biofilm & CFU

Materials:

- Sterile LB
- 14 ml bacterial culture tubes with lids
- Falcon tubes 15 ml & 50 ml
- Pipette tips
- multichannel pipette
- Sterile Double LB (2x concentrated LB with half the water volume)
- DPBS -$CaCl_2$, -$MgCl_2$ (Gibco Cat. No. 14190-094)
- 24-well plate with lid (flat bottom)
- LB agar plates
- Compounds for testing at desired concentration in LB or water
- Bacterial strain *E.coli* AR3110 (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 (can use tip instead of toothpick) and allow to incubate overnight (ON) at 37°C.

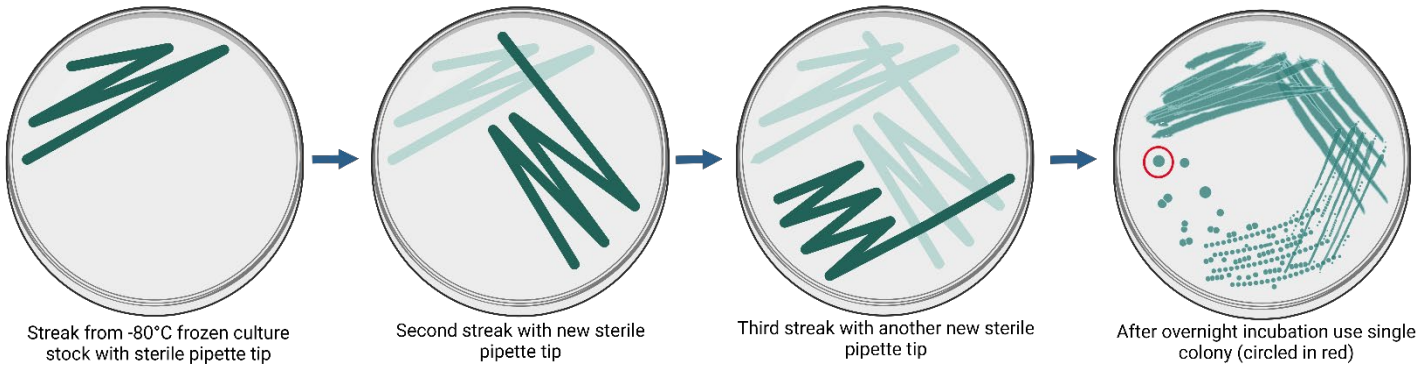

Figure 1: Single colony streak out method

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 to mid-exponential phase (OD_{600} 0.5-0.6) (approx. 3 hours, measuring OD_{600} every 30 min after 2 hours mark) The doubling time of *E. coli* is ~30 min at 37°C. Confirm that the negative control LB is still clear and sterile.

Create a stock bacterial solution* of with the OD_{600} of 0.02 by using the following formula to calculate the proper dilution:

$C1 \times V1 = C2 \times V2$, where C1 is the initial concentration, V1 is the initial volume, C2 is the final concentration and V2 is the final volume.

If the compound is in solution in water than use double LB for creating the inoculating bacterial stock.

To the 24-well plate, add 250µL of compound, at least 3 replicates for each compound. Then add 250µL of 0.02 OD_{600} bacterial stock so that the final concentration will be 0.01 OD_{600}. Make sure to vortex bacterial stock right before adding to wells. Pipette up and down to mix thoroughly with compound.

Make sure to include controls of compound with no bacteria (250µL compound + 250µL (double) LB) and bacteria with no compound (250µL water/LB + 250µL bacterial stock), at least 3 replicas for each control.

Example of experimental set-up in the 24-well plate:

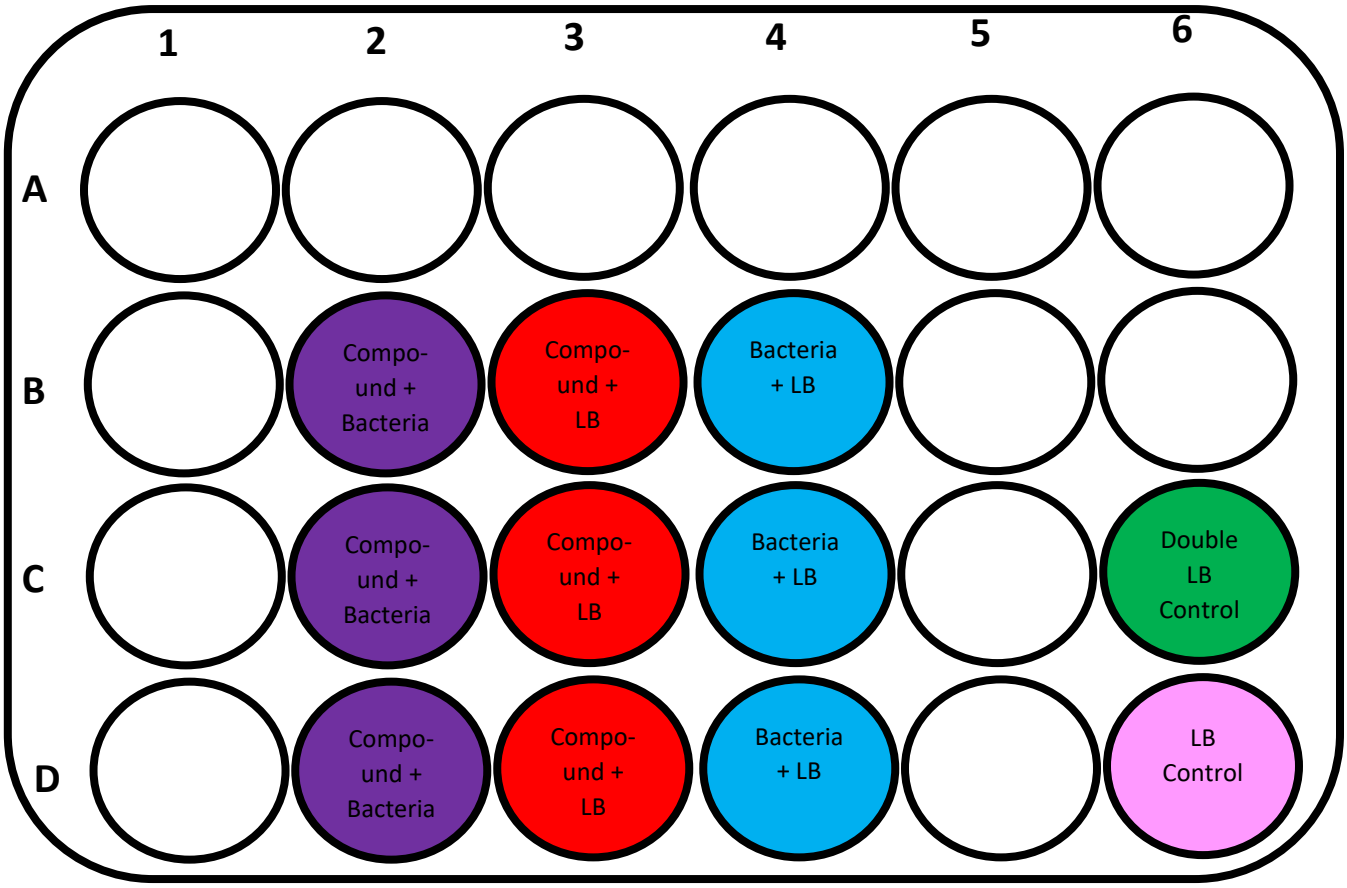

Figure 2: 24-Well Plate Set-Up

Cover plate and place in incubator at appropriate temperature in stationary condition for 48-72 hours.

Day 5 → Take out 20µL from each well for serial dilutions for CFU.

Fill a 96-well plate with 180µL PBS (-,-) buffer using multichannel pipette via a reservoir. Add 20µL of incubated compound-bacterial solution to row A, each well has a different replicate/compound, and mix thoroughly. Change tips between compounds.

Serially dilute 20µL of mixture from row A to H, changing tips between rows and mixing thoroughly by pipetting up and down at least 10 times per well. May use multichannel pipette. Can throw away last 20µL from row H, to ensure same volume in each of the wells. (See diagram below).

Meanwhile dry LB-agar plates thoroughly on clean bench.

Mark LB-agar plates with a line using marker to be able to orient the lid.

Plate 20µL drops onto LB-agar starting from H to E, can use the same tip for each compound when increasing the dilution. Place lid on top of plate and trace where the droplets were placed onto the lid and label each drop. Carefully allow the drops to fully dry in clean bench. Once dry cover and place upside down to grow ON in the incubator at 37°C.

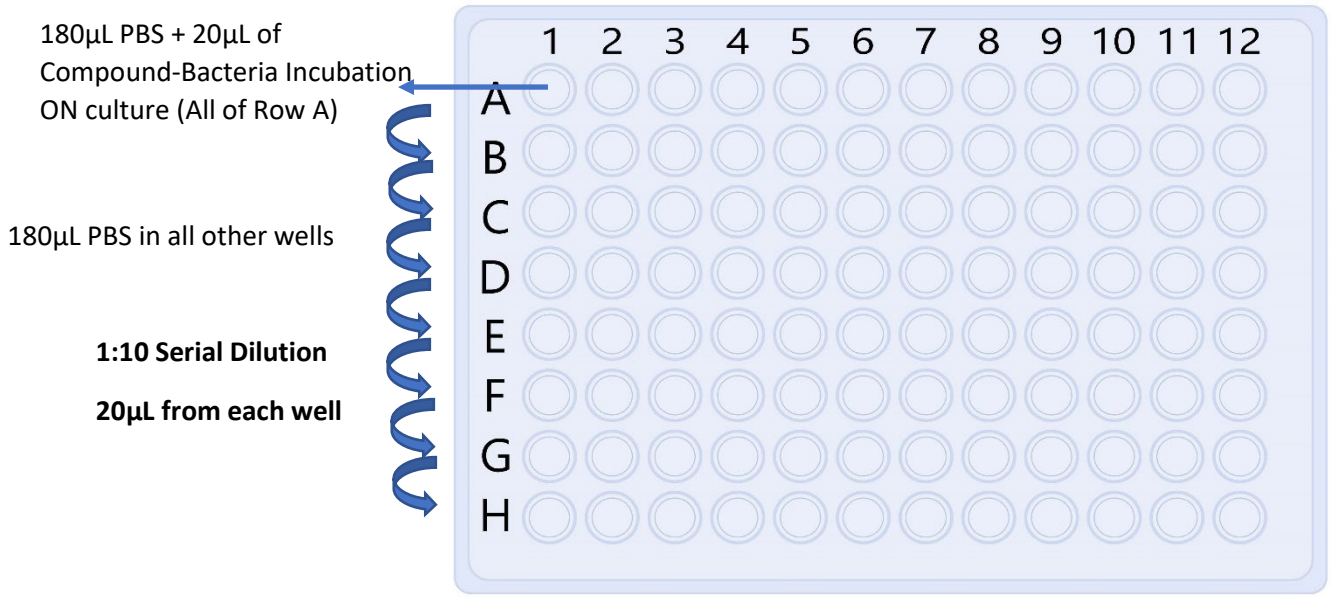

Figure 3: 96-Well Plate Set-Up of Serial Dilution

Using vacuum in hood suck out all of the remaining liquid in wells, carefully not to disturb the biofilm. Add 500µL 0.1% Crystal violet (CV) solution and allow to react for 10 min. Suck out CV and wash biofilms with 500µL PBS and suck out liquid. Add 500µL 96% ethanol to each well and dissolve the biofilm. Measure the OD by plate reader to get the biomass of the biofilm and the inhibition rate.

Day 6 → 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 (20µL) and dilution factor:

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

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

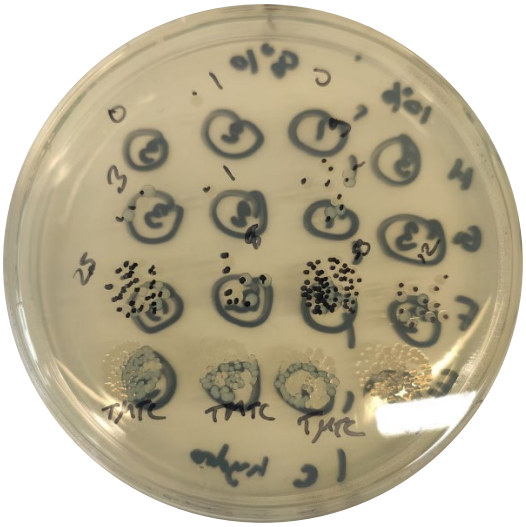

Figure 4: LB Agar plate after colonies have formed and counted with marker