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Version 1

S. aureus biofilm removal multi-assay V.1

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We are still developing and optimizing this protocol

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Abstract

The following protocol describes culture and treatment of the S.aureus-based biofilm and techniques that can be used to assess its eradication/removal. The protocol covers basic preparation and three techniques, namely: growth control, colony forming assay, and crystal violet staining.

Troubleshooting



Biofilm culture

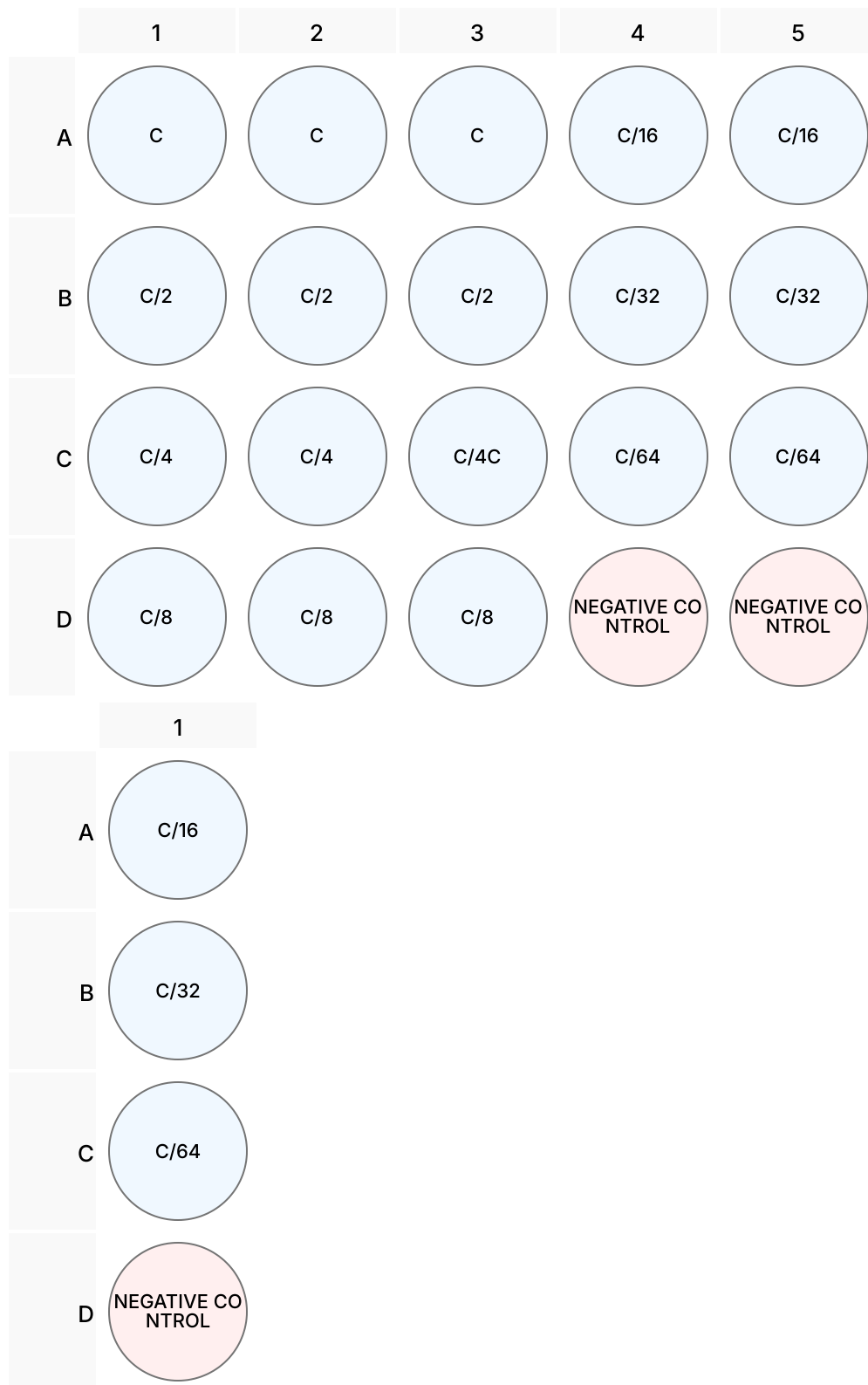
1d 0h 5m

- 1 Culture bacteria strains in **TSB** at 35 °C for approximately 04:00:00 in order to reach mid-log phase (OD₆₀₀ 0.5-0.6). 4h
- 2 Pellet the cells by centrifugation (4500 rpm, 20°C, 00:05:00) then wash twice with potassium phosphate buffer (**PPB**; [M] 100 millimolar (mM) , 7). 5m

For washing, redisperse and repeat the centrifugation. First two washes with 3 mL of **PPB**, then redisperse in 3 mL of **PPB**
- 3 Adjust bacterial suspensions to 10⁷ CFU/mL in **PPB** (OD₆₀₀= 0.01, ref: **PPB**).
- 4 Initiate biofilm formation in 24-well plate by covering surface with 1 mL of the adjusted cell suspension, and incubate the plate at Room temperature for 02:00:00 to allow the bacterial adhesion. Remove the content of the wells after this time. 2h
- 5 Cover the wells with 1 mL of **TSB**, and put a protective film before putting the lid to minimize the evaporation. Incubate the bacteria for at least 18:00:00 18h

Treatment of the biofilm

- 6 Prepare your substance in either **PBS** or **NaCl** ([M] 9 Mass Percent) by serial dilution ranging from C to C/(X²) where X is the number of concentrations tested. A 24-well plate can hold 7 concentrations + negative control.
- 7 After the biofilm matured, remove the 24-well plate from the incubator.
- 8 Gently remove the **TSB** using either pipette or vacuum aspirator. After removing medium from a cell, immediately wash it with **PPB**, **PBS** or **NaCl** two times. After last washing add 1 mL of your sample. Repeat for concentration in triplicate. Same as before, apply protective film before incubating the plate.







- 9 Treat the biofilms at suitable temperature and time. This two factors have to be estimated prior to the treatment, for example by means of crystal violet staining (see below).



- 10 After the time has passed, remove the plate, and start removing the content of the well. After each removal, wash the remaining biofilm three times with either **PBS**, **NaCl** or **PPB**. Remove remaining liquid after and perform on of the techniques described below.

Growth control



- 11 Add  0.5 mL of **TSB** to each well and indubate the plate at  37 °C for  06:00:00  08:00:00

14h

- 12 Transfer the supernatant to 96-well plate and measure OD₆₀₀, dilute with **NaCl** if necessary.

Colony forming assay



4h

- 13 Add  1 mL of either **PBS**, **NaCl** or **PPB** to the well with a pipette, directly onto the biofilm, so the thrust of the liquid damage the biofilm.
- 14 Scratch the bottom of the well with a pipette tip and transfer the content to eppendorf tubes. Vortex-shake eppendorfs for couple of seconds or until reaching homogeneity.
- 15 Serially dilute the content of the eppendorf tubes in 96-well plate.
- 16 Plate 20µL of the content of the wells on the **BHI** medium prepared in the square Petri dish.
- 17 Incubate Petri dish  Overnight
- 18 Next day, count the colonies and asses the CFU/mL or CFU/cm².

4h










Crystal violet staining + brightfield microscopy

1h 30m

- 19 Dry the biofilms for  00:30:00 in  Room temperature

30m



- 20 After that time, fix the biofilms by adding  200 μL of methanol to each well. Wait  00:15:00 and remove any remaining methanol. 15m
- 21 Add  250 μL of  0.06 Mass Percent solution of crystal violet to each well and wait  00:15:00 . 15m
- 22 Remove any non-absorbed dye by washing gently stained biofilms with either **PBS, NaCl** or **PPB**.
- 22.1 At this moment you can perform brightfield microscopy of the biofilm to image better its structure.
- 23 Add  1 mL of acetic acid to the wells and gently shake  120 rpm, Room temperature them horizontally for  00:30:00 . 30m
- 24 Remove  200 μL of the solubilised pigment with a pipette and transfer it to 96-well plate and dilute serially with water.
- 25 Record the absorption of the wells at **570 nm**. The percent of the remaining biomass is calculated by dividing the A_{570} of the sample to A_{570} of the control.