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R2-SOP for working with Staphylococci

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Mariana Blanco Massani¹

¹University of Innsbruck

Mariana Blanco Massani: Marie Skłodowska-Curie IF, NanoBioRS-101025065



marianablanco.m

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We use this protocol and it's working

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Disclaimer

Dissemination level: (Confidential until publication of results from NanoBioRS-101025065).

Abstract

The protocol presents a stepwise guideline for the determination of the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum antibiofilm concentration (MAC) of antimicrobials by the microdilution method. The work is structured into sequential experimental steps, each carried out on a different day to help the trainee organize the experimental setup.

The application of the same protocol for studying interaction of antimicrobials is described at the end of the document. Three independent replicates are recommended to validate the results.

Image Attribution

Figure 1. Experimental setup for studying interaction of antimicrobials.



Guidelines

1. Scope:

This document was created in order to facilitate knowledge transfer between the ER (Mariana Blanco Massani) and the host institution (University of Innsbruck- UIBK, Austria) and the host for secondment stage (Jagiellonian University-JU, Poland) as part of NanoBioRS-MSCA IF action (NanoBioRS-101025065).

The publication of this document is in line with data sharing as delineated in the data management plan of NanoBioRS (D2.1-ORDP-NanoBioRS).

The available bacterial species to transfer knowledge about are *Staphylococcus aureus* ATCC 25923 and *S. epidermidis* 9142, but this protocol can be applied to any mesophilic strain able to produce biofilm under similar conditions as those developed here. Examples are *Escherichia coli*, *Pseudomonas aeruginosa*, provided that the growth medium is suited to the metabolic capabilities and biofilm formation for each species, more information can be found in <https://beta.bacdiver.dsmz.de/api-test-finder>.

The expertise transferred by the ER was not available before at the Drug Delivery and Powder Technology Group (DDPT-UIBK). The methods described here were published in (Blanco et al., 2020; Blanco Massani et al., 2018, 2024; Verdi et al., 2020). Additionally, an adaptation of the methodology developed by others (Haney et al., 2021; Olsen et al., 2018) was applied with some changes in three scientific articles from Blanco Massani *et al.* that are under review at the moment of this document preparation.

Materials

Materials and methods — Day 1 (preparation of stock plates)

Materials required:

- Glycerol stock of Staphylococci
- Sterile loop
- Autoclave
- Tryptic Soy Broth (TSB) supplemented with agar 1.5% w/v (TSA)
- Sterile petri dishes
- Laminar flow BSL2
- Incubator

Day 2 (Quality Control of cells)

Materials required:

- Sterile mannitol red agar
- Sterile loop
- Laminar flow BSL2
- Incubator

Day 2 (Activation of bacteria and Broth microdilution method)

Materials required:

- A set of 2-fold dilutions of the antimicrobial of interest. For known antibiotics the concentration to be tested can be estimated based on the breakpoints published at https://www.eucast.org/clinical_breakpoints.
- TSA plate stock of staphylococci that met the QC requirements
- Filter sterilized glucose¹
- Liquid medium: TSB or TSB+glucose¹
- Autoclave
- Sterile petri dishes, 96 well plate, tips and Eppendorf tubes, water

Day 3 (MIC determination)

Materials required:

- Autoclave
- Sterile TSA plates
- Sterile tips
- Spectrophotometer
- Laminar flow BSL2
- Incubator

Day 4 (MBC determination)

Materials required:

- Laminar flow BSL2
- Incubator
- Sterile tips, NaCl 0.85% (physiological solution)



Day 5 (MAC determination)

Materials required:

- Laminar flow BSL2
- Sterile tips and water
- Violet crystal stock solution 2% (w/v)
- 33% glacial acetic acid
- Spectrophotometer

Interaction of antimicrobials

Materials required:

- Sterile tips, water, TSB or TSB+glucose¹
- Laminar flow BSL2
- Incubator
- Serial 2-fold dilutions of the antimicrobials of interest.
- Spectrophotometer

Safety warnings

! Check the BSL of the strains used. Work following the corresponding guidelines.

Before start

2. Bacterial cells and growth conditions.

Staphylococcus aureus ATCC 25923 (methicillin sensitive, biosafety level 2, BSL2) and *Staphylococcus epidermidis* 9142 (biosafety level 1, BSL1) were provided by the Biofilm Lab (Experimental Orthopaedics, Department of Orthopaedic Surgery, Medical University Innsbruck). Bacterial stocks of staphylococci were stored at -20 °C in glycerol and expanded in TSB at 37 °C under shaking (200 rpm).



Day 1 (preparation of stock plates) — Methods

- 1 Prepare and autoclave TSA according to the manufacturer indications.
- 2 Pour ca 20 ml in petri dishes and let the agar solidify in a sterile environment with the lid slightly open.
- 3 Using a sterile loop, streak the bacterial strain of interest from the glycerol stock onto the solidified TSA plate.
- 4 Incubate overnight at 37 °C for 24–48 h.
- 5 Store the petri dish as a working stock for not more than 1 month in the fridge at 4 °C.

Day 2 (Quality Control of cells) — Methods

- 6 Streak some colonies from the TSA grown plate onto Mannitol red agar.
- 7 Incubate 48 h at 37 °C and inspect the results obtained.



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Expected result

Staphylococcus aureus change the color of the medium from pink to yellow due to Mannitol fermentation. *S. epidermidis* is growing without changing the color of the medium. *Micrococcus luteus* strains that can be environmental contaminants, and which colonies can be confused with staphylococci do not grow in this medium. Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS) can be used as a reliable method for additional QC.

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**Note**

Perform QC of bacteria each time that they are streaked from the glycerol stock.

Day 2 (Activation of bacteria and Broth microdilution method) — Methods

- 10 Inoculate 3 colonies in 3 ml of TSB and incubate overnight at 37 °C under shaking (200 rpm).
- 11 Make a dilution 1:5 of the grown culture and incubate for 1 h at 37 °C under shaking (200 rpm).

Note

This step is performed to ensure that bacteria are at the Log phase of growth for the experiments, which ensures the reproducibility of results.

- 12 Dilute the sample under sterile conditions using TSB or TSB+glucose¹ to achieve a McFarland of 0.5, and then make a dilution accordingly to reach 10⁵ Colony Forming Units (CFU)/ml (inoculum).
- 13 Make 2-fold dilutions of the antimicrobial agent of interest using sterile water.
- 14 Add 100 µl of the antimicrobial and its 2-fold dilutions on wells of the 96-well plate in technical duplicates. Include a negative growth control (100 µl sterile medium/100 µl sterile water) and a positive growth control (100 µl inoculum/100 µl sterile water) in the 96 well plate.
- 15 Add 100 µl of the inoculum containing 10⁵ CFU/ml and incubate overnight at 37 °C under static conditions for MIC and MBC determination only.
- 16 If antibiofilm properties of the antimicrobial are to be determined, the setup should include conditions promoting biofilm formation¹.
- 17

**Note**

¹ Suggested conditions for promoting biofilm development by staphylococci are TSB supplemented with 1% w/v glucose (TSB+glucose) cultured 37°C with shaking at 200 rpm (Blanco Massani *et al.* 2024)

Day 3 (Minimum inhibitory concentration, MIC determination) — Methods

- 18 Read absorbance of the 96 well plate at 620 nm.
- 19 Select the dilutions that show the same absorbance as the negative growth control (no turbidity).
- 20 The MIC is defined as the lowest concentration of antimicrobial showing no turbidity under the assessed conditions.
- 21 Inoculate on TSA agar plates 100 µl from the wells showing no turbidity. Spread the inoculum using a Drigalki spatula or ColiRollers
- 22 Incubate the plates for 24–48 h.

Day 4 (Minimum bactericidal concentration MBC determination) — Methods

- 23 Inspect the plates and count cells grown to calculate the corresponding CFU/ml.

Note

This calculation helps determine whether the observed effect is bacteriostatic or bactericidal, depending on the degree of inhibition (Verdi *et al.* 2020).

- 24 The MBC is defined as the lowest concentration showing no viable bacteria, which corresponds to 99.9% of inhibition (Blanco Massani *et al.* 2018)
- 25 Carefully rinse the well plate once with sterile physiological solution to remove planktonic bacteria for MAC determination



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Safety information

All the experiments involving *S. aureus* have to be performed in a BSL2 facility. Waste-autoclave the agar plates and all the materials that were in contact with bacteria and follow the biosafety guidelines provided by the BSL2 facility.

Day 5 (Minimum antibiofilm concentration, MAC determination) — Methods

- 27 Prepare violet crystal 0.1% w/v by diluting the stock 2% w/v solution with water.
- 28 Add 200 µl of violet crystal to each well and incubate for 15 min at 37 °C under shaking (200 rpm).
- 29 Carefully rinse once with water and dry the plates at 37 °C
- 30 Add 200 µl of 33% glacial acetic acid to each well and incubate for 15 min under shaking (200 rpm).
- 31 Read absorbance at 593 nm
- 32 Normalize the absorbance data obtained
- 33 The MAC is defined as the lowest concentration showing less than 40% of normalized absorbance under this conditions (Olsen *et al* 2018).
- 34

Safety information

Waste-autoclave all the materials that were in contact with bacteria and follow the biosafety guidelines provided by the BSL2 facility.

Interaction of antimicrobials — Methods

- 35 Make 2-fold dilutions of the antimicrobial agent of interest using sterile water.
- 36 Distribute the 2-fold dilutions of the different antimicrobials as described in **Figure 1**. For each concentration combination, 50 μ l of antimicrobial A and 50 μ l of antimicrobial B should be added to each well. Include in the setup the dilutions of each separated antimicrobial to determine the MIC in the same experimental replicate.
- 37

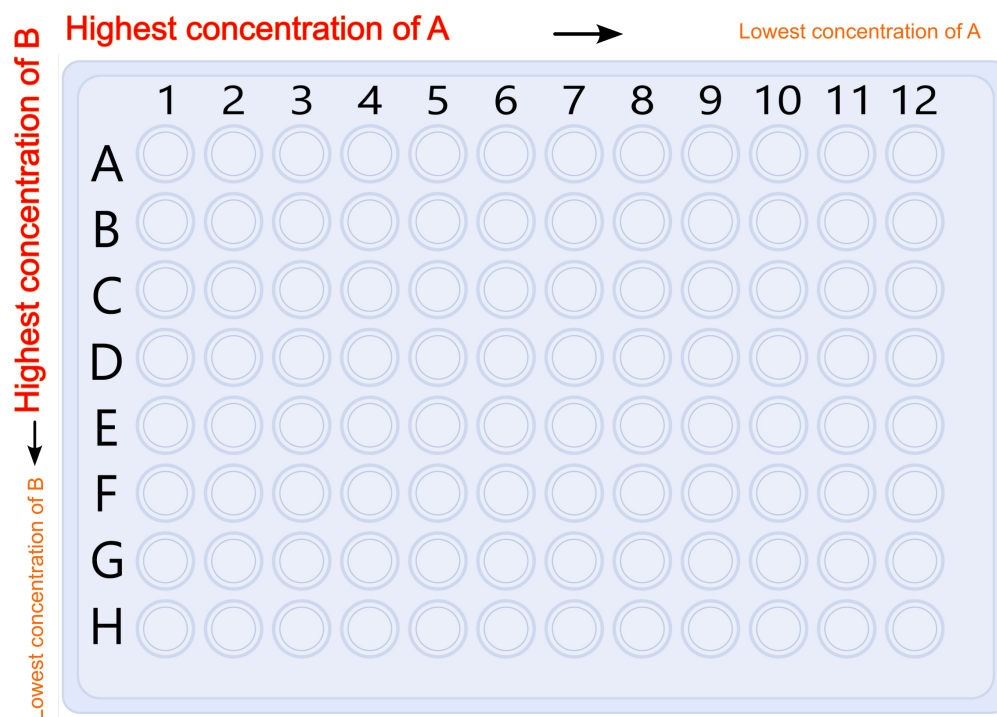


Figure 1. Experimental setup for studying interaction of antimicrobials.

- 38 Inoculate the plates, incubate and read absorbance as described in the MIC determination-Method.
- 39 The concentrations showing no growth can be used to assemble isobolograms for the binary combinations (Verdi *et al.* 2020).
- 40 Calculate the fractional inhibitory concentration (FIC), which is given by the minimum inhibitory concentration (MIC) of an antimicrobial when combined in a mixture, divided

into the MIC of the single antimicrobial.

- 41 Synergistic interactions are defined as the combinations presenting bactericidal action at sub-MICs ($\Sigma\text{FIC} < 0.5$), thus falling below the additive effects curve of the corresponding isobologram (Verdi *et al.* 2020).
- 42 Bactericidal and antibiofilm effects of the mixtures can be determined using the same plate as described for MBC determination-Method and MAC determination-Method, respectively.

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Additional resources: <https://beta.bacdive.dsmz.de/api-test-finder>; https://www.eucast.org/clinical_breakpoints



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