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🌐 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Assays Using Broth Microdilution Method



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Protocol status: Working

We are currently using this protocol, and it's working. However, the protocol might be updated in the future if any steps need to be streamlined.

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Abstract

The presented protocol outlines a comprehensive assessment of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for bacterial cell cultures. These experiments are vital for screening bacterial susceptibility to antibiotics and substances with potential antibacterial properties. The protocol not only covers the necessary preparatory steps but also introduces the application of the widely recognized Gompertz model. The protocol ensures a smooth execution of the assessment through thorough preparation and step-by-step instructions. The user-friendly instructions provided enable researchers to easily follow the protocol, facilitating the implementation of the assessment. By adhering to the outlined procedures, researchers can acquire a deeper understanding of bacterial susceptibility, evaluate the efficacy of antimicrobial agents through MIC and MBC values, and contribute to the advancement of antibacterial strategies.

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Guidelines

The following protocol is optimised for substances that are soluble in water. However, we recommend to prepare a small portion of the solution of the investigated substance and mix it with three portions of Mueller-Hinton Broth medium to see if any precipitation or liquid-liquid phase separation occurs. If that is the case – addition of small amount of DMSO (up to 1%) may help to solubilise the substance. If this won't help, we recommend using different technique to assess MIC and MBC values.

The following media have been used in the protocol:

-  BD DIFCO™ Mueller Hinton Broth 500g **Becton Dickinson (BD) Catalog #275730** – MHB
-  BD DIFCO™ Mueller Hinton Agar 500g **Becton Dickinson (BD) Catalog #225250** – MHA

The protocol was optimised and tested using following strains:

- *S. aureus* 43300 (ATCC)
- *P. aeruginosa* 15442 (ATCC)
- *E. coli* K-12 (ATCC)

This protocol can be easily adapted to other strains of bacteria, however, make sure to use suitable media, if the investigated strain is not growing on the MHA/MHB. Bare in mind, that use of MHA/MHB is in accordance with CLSI guidelines; hence, any changes have to be indicated when publishing the results.


Gompertz model file was adapted from OriginPro forums and was originally posted by the user YimingChen.

Materials

- Standard 96-well microplate
- Pipettes
- Square Petri dishes with MHA
- Microtube (a.k.a EppendorfTube®)
- Trough (optional)
- Multichannel pipette (optional)
- Sealing safety film for microplate (optional)

Troubleshooting

Safety warnings

-  All the manipulations with handling live bacteria should be executed using the biosafety cabinet at all times unless local regulations state otherwise.






Before start

Be sure to read the protocol fully, before starting any manipulation. Be sure that all the equipment needed is working properly and you have enough time to do all the manipulations. If it is your first time doing this, be sure to plan more time, as usually first times take longer. Be sure that both you and environment is well protected and your laboratory regulations allow for manipulation with bacteria.

For a single test, that consist of a triplicate you will need around 4 mL of MHB, however, it is recommended to have much more prepared (preferably around 20 mL) in case any mishaps occur. You have to have an inoculated medium on a Petri dish with discrete colonies already prepared.



Pre-preparation of stock colony

- 1 Take the inoculated Petri dish and transfer one colony to a tube with approx.  3 mL of fresh **MHB**.
- 2 Incubate the tube for around  03:00:00 (or more, depending on the strain and the size and age of the colony used) at  37 °C .

3h

Note

Stock colony can be prepared in the morning and be worked with in the early afternoon. It can also grow overnight (to the late stationary phase) and be diluted (at least by 1:50 or 1:100) with the fresh **MHB**. In case of the second scenario, it will take 2-3 h to reach the optimum OD₆₀₀ (around 0.1). If you have any concerns refer to CLSI M100 guidelines.

Preparation of diluted standardized inoculum



3

Note

This part should be done only when the 96-well microplate is ready to be inoculated.

Prepare **standardized inoculum** by first measuring the OD₆₀₀ of your **stock colony**. If the value of the OD₆₀₀:

- Is greater than 0.1, dilute the culture with **MHB** to reach a value of 0.1,
- Is between 0.09 and 0.1 you can move to a next step,
- is below 0.09 put the culture back and incubate it for 15-30 minutes more.

- 4 Pour  10 mL of **MHB** into a trough and add  100 µL of the **standardized inoculum**. Mix it by flushing couple of times with a pipette.

Note

 10 mL of **diluted standardized inoculum** is enough to inoculate 16 rows.

Preparation of 96-well microplate

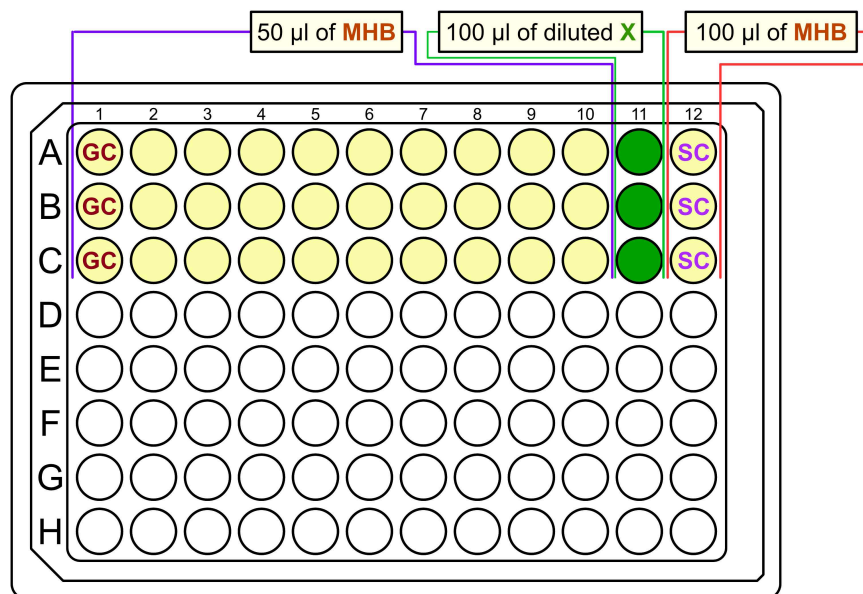
15m

- 5 Dissolve tested substance (**X**) in **MHB** at twice the maximum concentration for the test.

Note

E.g., if you want to test the concentration series of **X** starting from **100 µg/mL** then you need to prepare the stock solution of tested substance in the concentration of **200 µg/mL**. Adjust the total volume of dilution to your needs. A **100 µL** is needed for each row.

- 6 Pour around **10 mL** of **MHB** to the trough and:
1. Add **50 µL** of **MHB** to each well in **columns 1-10** (growth control, **GC (1)**+ serial dilution (**2-10**)),
 2. Leave wells in **column 11 empty** (highest concentration of serial dilution),
 3. Add **100 µL** to the wells in **column 12** (sterility control, **SC**).

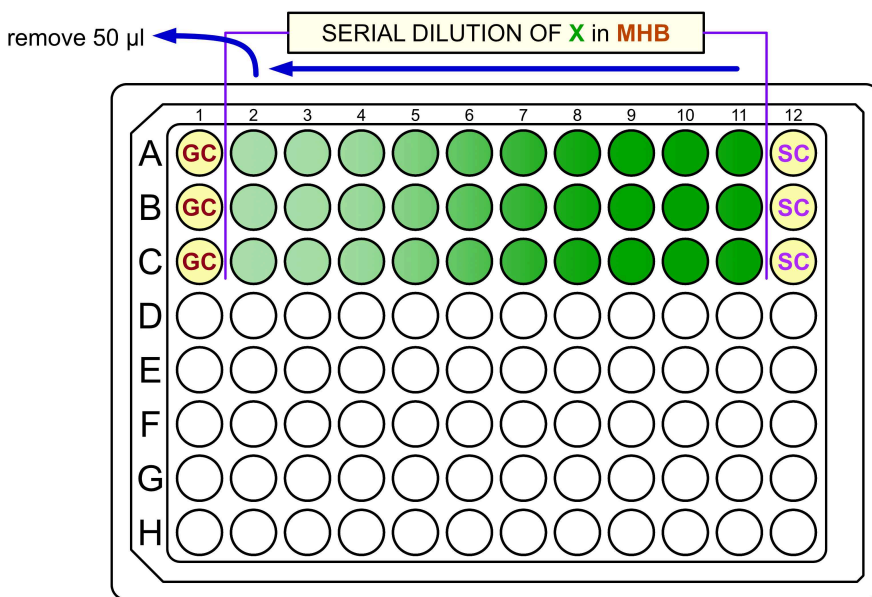


Typical microplate layout for this step.

- 7 Add 100 μL of the just prepared solution of **X** diluted in **MHB** to the wells in **column 11** and remove and pass 50 μL to next well until reaching wells in **column 2**,

Note

This will result in the wells in **column 2** having 100 μL . Remove 50 μL of the liquid content from these wells and discard it.

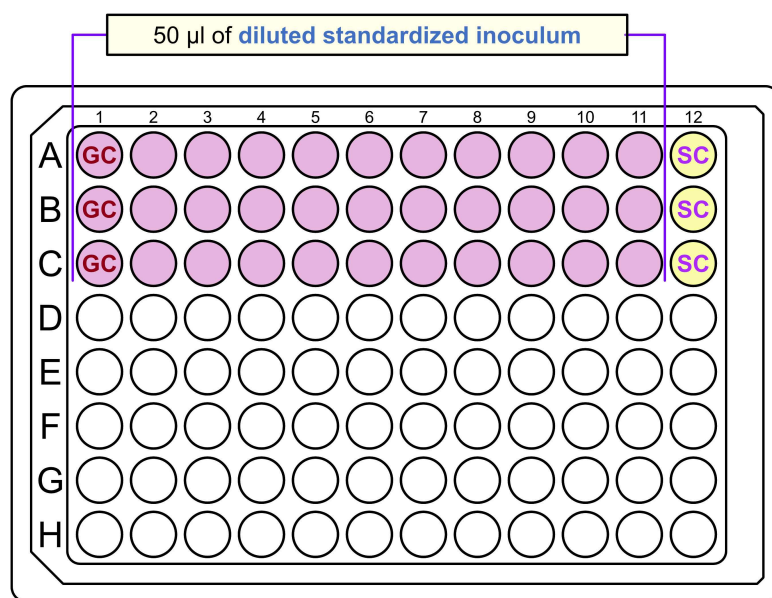


Serial dilution of **X** on the microplate.

- 8 Using multichannel pipette add 50 μL of **diluted standardized inoculum** to each well of **columns 1-11**. For more information [go to step #3](#) .

Note

The **standardized inoculum** is equivalent to [M] 10^8 CFU/mL . After 1:100 dilution in **MHB** the concentration is [M] 10^6 CFU/mL . Taking 50 μ L of the diluted bacterial suspension and adding it to all the wells of **columns 1-11** results in a final concentration of approx. [M] 5×10^5 CFU/mL . Be sure that the time needed to prepare and dispense the OD₆₀₀-adjusted bacteria solution do not exceed 00:15:00 .



Addition of the diluted standardized inoculum



- 9 Put a protective film on that-prepared well plate. Cover it with a lid. Put a name and/or other details on the side of the plate. Avoid leaving any marks on the lid.
- 10 Incubate the plate at 37 °C Overnight .

15m


Minimal inhibitory concentration (MIC) assessment

15m

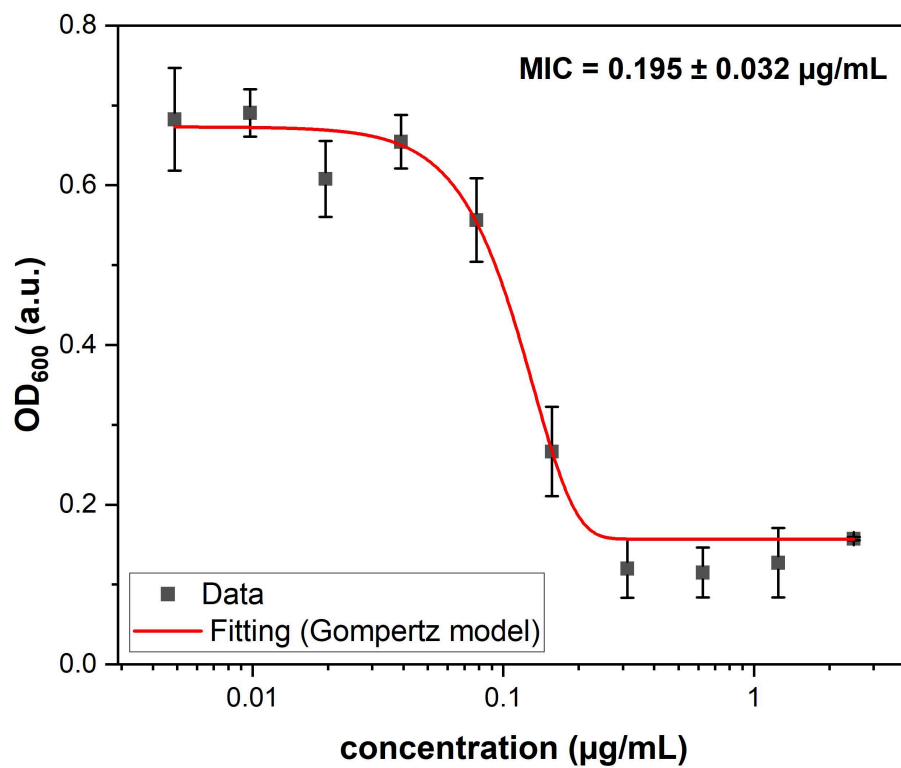


- 11 After the incubation take the microplate out of the incubator. Remove the lid from the plate and protective film if applied and let it cool down in  Room temperature for  00:15:00 .
- 12 After that time put the microplate into the plate reader and read OD₆₀₀ values of the wells' content.
- 13 Gather the data, and using plotting program such as Prism, OriginPro, Excel or Kaleida plot the data and fit it using modified Gompertz model.

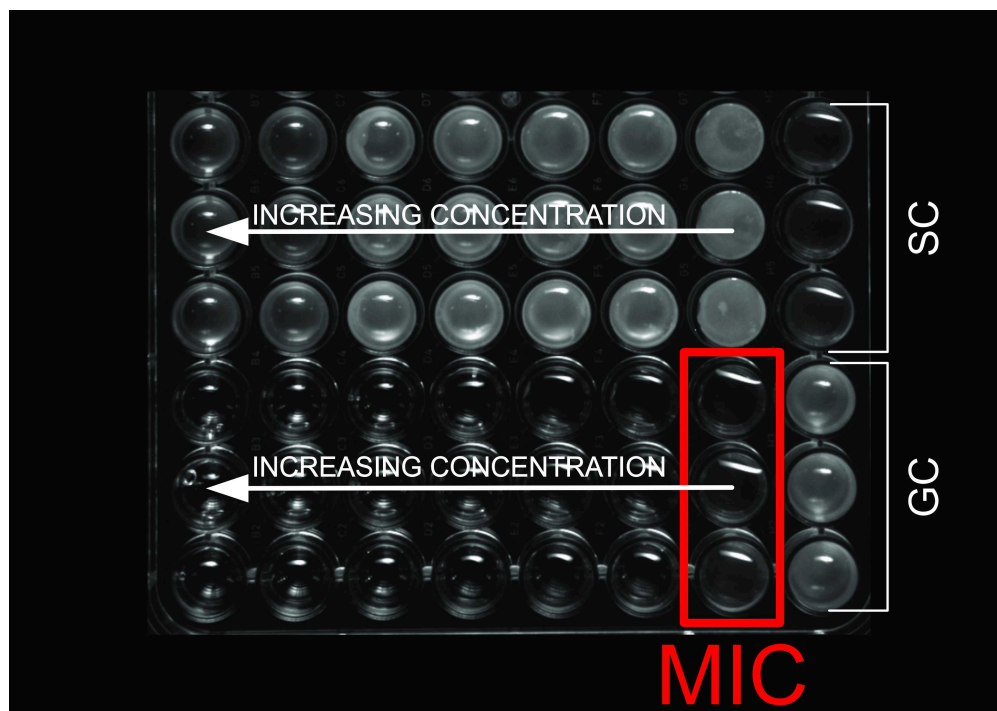
Note

MIC value lies on the intersection of the lower part of the jump with the jump slope (**example**). A OriginPro model file can be accessed here:  Gompertz - MIC.fdf

Alternatively – MIC value can be assessed visually. In that case MIC value is where the no turbidity is observed.



Example of data modelled using Gompertz model (OriginPro).

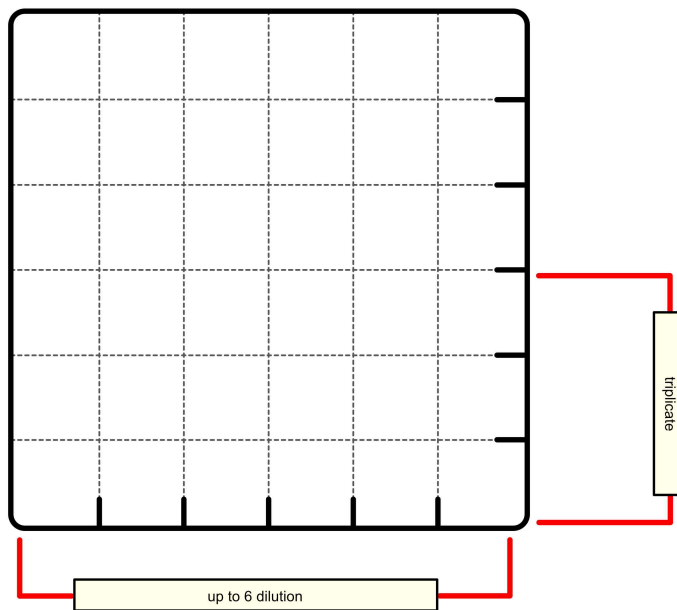


Visual assessment of MIC value

Minimal bactericidal concentration (MBC) assessment

15m

- 14 Transfer 100 μL of the content of the wells where no turbidity was observed to a separate 96-well microplate and dilute it ten fold serially with the solution of **NaCl** [M] 9 g/L
- 15 Plate 20 μL of the content of the wells directly onto square Petri dish/dishes with **MHA** and incubate Overnight



Possible maximum plating area of square Petri dish (side of. ca 120 mm).

- 16 Next morning count the colonies on the plates.

Note

You can omit the dilution and counting, and directly plate 20 μL of the content of non-turbid wells after MIC assessment. MBC value is, where no growth was observed. You can use this CFU calculator to count the surviving colonies that are between MIC and MBC values: CFU counter_ver_6.xlsm



Protocol references

Guidelines:

CLSI M07-A10 Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 10th Edition

CLSI M100 Performance Standards for Antimicrobial Susceptibility Testing, 33rd Edition

Gompertz Model:

https://my.originlab.com/forum/topic.asp?TOPIC_ID=47761 (accessed: 17/06/2023)

Example of use:

Elizaveta Sviridova, *et al.*, *Surface modification of carbon dots with tetraalkylammonium moieties for fine tuning their antibacterial activity*, Biomaterials Advances, Volume 134, 2022, 112697,

<https://doi.org/10.1016/j.msec.2022.112697>.