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Standard Operating Procedure for Determination of the Minimum Inhibitory Concentration (MIC) of Different Antimicrobial Agents Against Different Bacteria

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Alanoud T Aljasham¹, Mashal M. Almutairi^{1,2}

¹Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia;

²The Chair of Vaccines Research for Infectious Diseases, King Saud University, Riyadh 11495, Saudi Arabia

Dr. Mashal Alshazi's Lab...



Alanoud T Aljasham

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We use this protocol and it's working

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Abstract

This standard operating procedure (SOP) describes the standardized laboratory procedure for performing the MIC experiment.

Guidelines

Scope:

This procedure applies to all antimicrobial agents and bacteria when performing MIC experiment.


Definitions and Acronyms:

	A	B
	Term/Acronym	Definition
	SOP	Standard Operating Procedure
	MH	Mueller Hinton
	OD 600	Optical density at a wavelength of 600 nm



Materials

Solutions and Materials:

- MH broth media
- Antimicrobials
-  96 Well Round Bottom Non-treated Plate Sterile **Cell Treat Scientific Products Catalog #229590**
- Medium Reservoir
- 1.5 ml Eppendorf tubes.
- 15 ml tubes
- Ice
- Spectrophotometer cuvettes

Instrument:

- Spectrophotometer
- Incubator
- Electronic balance
- Vortexer
- Minicentrifuge
- Plastic box
- Different pipettes

Troubleshooting



Safety warnings



Safety Precautions:












- All the MIC procedures should be taking place in a designated area at the lab.
- Before and after processing of the samples, the workstation should be disinfectant with 70% ethanol or 10 % bleach, and all the tools used during the processing must be sterilized.
- A properly fastened laboratory coat, long pants/skirt, closed toed shoes and gloves are required when working with clinical isolates.
- After handling any contaminated samples, gloves must be removed, and hands must be washed properly.
- All contaminated materials and gloves must be discarded in the biohazard containers.
- Prior to disposal, decontaminate all waste with 70% ethanol or 10% bleach.

Before start








Before starting, the work area should be sterilized with 70% ethanol, and all the instruments that will be used during the experiment should be sterilized.



Antimicrobial Agents Stock Solution Preparation

- 1 Set the electronic balance to mg unit
 - 2 Place an empty sterilized 1.5 Eppendorf tube onto the balance and zero it.
 - 3 Add the desired amount of the antimicrobial powder into the Eppendorf tube. 
 - 4 Add  1 mL of the recommended solvent (depending on the antimicrobial compound) in the same tube.  
- ### Note
- Vortex until the antimicrobial powder completely dissolved in the solvent.
- 5 Then, give it a quick spin down. 
 - 6 Make  100 μ L aliquots and store them at  -20 °C . 
 - 6.1 For example: if you want to prepare [M] 20 mg/mL of ampicillin, weigh  20 mg of ampicillin in a 1.5 Eppendorf tube and then add  1 mL of H₂O (the solvent). 

Bacterial subculturing from glycerol stock

- 7 To begin with, using sterilized pipette tip or sterilized loop, inoculate small amount of the bacterial stock (stored at  -80 °C) into MH broth medium in 1.5 Eppendorf tube. Incubate it at  37 °C shaking  Overnight .    



8 Next Day, prepare 1:50 or 1:100 dilution from the bacterial inoculum in fresh MH medium and incubate it for 02:00:00 - 03:00:00 until the OD₆₀₀ reach (0.3 – 0.7).

5h



8.1 For example, if you want to make 3 mL of the 1:100 dilution, divide the final volume by the dilution factor (e.g., 3ml (3000ul)/100) = 30ul.



8.2 Then, transfer 30 µL of the Overnight bacterial culture into a 15ml tube containing 3 mL of fresh MH broth and incubate for 02:00:00 - 03:00:00 at 37 °C .

5h



8.3 Prepare the 96-well plate while waiting for the optimal growth of bacteria at OD₆₀₀ 0.3 – 0.7.

96-Well Plate Preparation:

9 Label the plate and its lid with the name of the bacteria, antimicrobial agents, date and the name of the person performing the MIC experiment.

10 Using a multi-channel pipette, dispense 50 µL of MH broth into each well of the 96-wells (except for column number 12 you need to dispense 100 µL), use the same tip for one plate.



11 Add two-fold the required concentrations of the antimicrobial agents to the column no. 12.



11.1 For example, if highest desired concentration is 256 µg/ml , add to 512 µg/ml .



11.2 Use the following equation to calculate the needed volume of the antimicrobial agent:

$$C_1 \times V_1 = C_2 \times V_2$$

Where

C₁= The antimicrobial stock concentration



V_1 = The volume that is needed to be taken from the antimicrobial stock concentration to achieve the required antimicrobial concentration in column no.12

C_2 = The required antimicrobial concentration in column no.12

V_2 = The total volume of the medium in column no.12

- 11.3 Follow this example to calculate the V_2 , if the antimicrobial agent stock concentration is





[M] 20 mg/mL and the highest desired concentration is [M] 256 $\mu\text{g/ml}$.

- 11.4 Then,

$$C_1 \times V_1 = C_2 \times V_2$$




20mg/ml $\times V_1$ = 512ug/ml $\times 100\mu\text{l}$




V_1 = 0.512mg/ml $\times 100$ / 20mg/ml = 2.56 μl .



- 11.5 A volume of  2.56 μL of antimicrobial agent will be added to the column no. 12, which contain  100 μL MH broth, and mix by pipetting up and down 3 times.  

Note

The concentration of antimicrobial agent in column no. 12 will be [M] 256 $\mu\text{g/ml}$.


- 12 Next, using a multi-channel pipette set at  50 μL , mix antimicrobial agent in the wells in column no. 12 by pipetting up and down 3 times without splashing and introducing air bubbles.  

- 13 Withdraw  50 μL from column no.12 and add them to column no. 11 (This makes column no. 11 a two-fold dilution of column no. 12, with antimicrobial agent concentration at column no. 11 of [M] 128 $\mu\text{g/ml}$). Mix up and down 3 times.  

- 14 Repeat the same procedure in step 13 by taking  50 μL from the corresponding column and add it to the preceding column until column no. 2. 






- 14.1 Discard the  50 μL from column no.2.



- 14.2 Column no. 1 should not contain any antimicrobial agent (just the  50 μL of MH broth you added from the starting of the MIC experiment).



Bacterial Preparation for MIC: Measuring OD600

- 15 After  02:00:00 -  03:00:00 of bacterial incubation at  37 $^{\circ}\text{C}$, measure OD₆₀₀ using the following steps: Turn on the spectrophotometer and wait until the automatic calibration is finished, make sure that the absorbance is set at 600nm.
- 16 Add  1 mL of MH medium only into a cuvette as a blank, then place the cuvette into the spec. and close the lid.
- 16.1 Press blank. The screen will give you a reading of the absorbance equals to 0.000.
- 16.2 Remove the cuvette and discard the MH medium from it.
- 17 Add  1 mL of the growing culture to the same cuvette and place back into the spectrophotometer. Press measure and record your reading.

5h



Bacterial Preparation for MIC: Bacterial Addition

1d 12h

- 18 After achieving the desired OD₆₀₀ of 0.3-0.7, dilute the bacterial culture OD₆₀₀ to 0.004, using the following equation:

$$C_1 \times V_1 = C_2 \times V_2$$

Where

C₁= The measured bacterial OD₆₀₀

V₁= The volume that is needed to be taken from bacterial culture to achieve the required bacterial OD₆₀₀ of 0.004

C₂= The required bacterial OD₆₀₀ of 0.004



V_2 = The required volume of the bacterial culture of OD_{600} of 0.004

18.1

For example, if the bacterial OD_{600} is 0.5



$$0.5 \times V_1 = 0.004 \times 5 \text{ ml}$$

We selected $V_2 = 5 \text{ ml}$ because the amount of bacterial culture for one plate is around 5 ml.

As $96 \text{ wells} \times 50 \text{ ul} = 4800 \text{ } \mu\text{l} \approx 5000 \text{ } \mu\text{l}$ (5 ml).

Therefore, $V_1 = 40 \text{ ul}$.

18.2

Next, transfer the calculated  40 μL of the growing culture to  4960 μL of fresh MH broth.



18.3

Mix well or vortex.




18.4

Then, quick spin down.



19

Divide the diluted bacteria into 8 Eppendorf tubes each with  625 μL . Therefore, each tube will be used for one row of the 96-well plate (to avoid cross contamination).



20

Dispense  50 μL of the bacterial cultural of the OD_{600} of 0.004 to all the wells.



Note

Use one Eppendorf tube and one tip for each row, start adding the bacterial culture from column no. 1 until column no. 12 (from zero to the highest concentration of antimicrobial to prevent the contamination of the wells by the highest concentrations of antimicrobial).

20.1

Mix the solution 1-2 times after each addition of the bacterial culture.



21

Cover the plate and place it in a wet and closed plastic box then incubate it for



18:00:00

at



37 °C



Overnight .

1d 12h





- 22 Next day, column no. 1 should show bacterial growth as a control (presence of turbidity).
- 23 Determine the MIC for each antimicrobial agent by finding the lowest concentration of the antimicrobial in each row that inhibit the visible bacterial growth (clear broth with no turbidity) and record the results.