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

Alanoud T Aljasham[1], Mashal M. Almutairi[1,2]

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

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

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Dr. Mashal Alshazi's Laboratory, King Saud University

Alanoud T Aljasham

ABSTRACT

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

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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

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MATERIALS TEXT

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

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 STARTING

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

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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.

Vortex until the antimicrobial powder completely dissolved in the solvent.

5 

Then, give it a quick spin down.

6 

Make **100 µL** aliquots and store them at **-20 °C** .

6.1 

For example: if you want to prepare **20 mg/mL** of ampicillin, weigh **20 mg** of ampicillin in a 1.5 Eppendorf tube and then add **1 mL** of H_2O (the solvent).

Bacterial subculturing from glycerol stock

7 

3h

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** .

5h

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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 OD_{600} reach (0.3 – 0.7).

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 5h

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** .

8.3 Prepare the 96-well plate while waiting for the optimal growth of bacteria at $OD6_{00}$ 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** .

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

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11.2

$$C_1xV_1 = C_2xV_2$$

Where

C_1= 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 **20 mg/mL** and the highest desired concentration is **256 µg/ml** .

11.4 Then,

$$C_1xV_1 = C_2xV_2$$

20mg/ml x V_1= 512ug/ml x 100ul
V_1= 0.512mg/ml x 100/ 20mg/ml = 2.56µl.

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.

The concentration of antimicrobial agent in column no. 12 will be **256 µg/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 

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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 **128 µg/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

5h

15 

After **02:00:00** - **03:00:00** of bacterial incubation at **37 °C** , measure OD_{600} 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 

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Add **1 mL** of the growing culture to the same cuvette and place back into the spectrophotometer. Press measure and record your reading.

Bacterial Preparation for MIC: Bacterial Addition 1d 12h

18

After achieving the desired OD_{600} of 0.3-0.7, dilute the bacterial culture OD_{600} to 0.004, using the following equation:

$$C_1xV_1 = C_2xV_2$$

Where

C_1= The measured bacterial OD_{600}
V_1= The volume that is needed to be taken from bacterial culture to achieve the required bacterial OD_{600} of 0.004
C_2= The required bacterial OD_{600} 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

$$0.5 \times V_1 = 0.004 \times 5\ ml$$

We selected V_2 = 5 ml because the amount of bacterial culture for one plate is around 5 ml.
As 96 wells x 50 ul = 4800 µl ≈ 5000 µl (5 ml).
Therefore, V_1= 40 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

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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.

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

1d 12h

Cover the plate and place it in a wet and closed plastic box then incubate it for **18:00:00** at **37 °C** **Overnight** .

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.

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