Minimum Inhibitory and Bactericidal Concentration Testing of Haloarchaea

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Background

Halophilic archaea are extremophiles that exhibit intrinsic resistance to multiple antibiotics due to their unique lipid membranes, efflux systems, and high-salinity adaptation. MIC and MBC assays are essential for assessing antimicrobial susceptibility, particularly in studies involving multidrug efflux pumps, biofilm resistance, and novel antimicrobials.

Unlike standard bacterial MIC/MBC protocols, testing in archaea requires:

- High-salt media (e.g., Payne's medium) for optimal growth
- Extended incubation times (~3 days) due to slow growth
- Modified endpoint determination (turbidity assessment, not OD600 alone)

This protocol adapts NCCLS standard microdilution methods for haloarchaea.

Reagent	Brand/Manufacture	Catalogue	Notes
_	r	No.	
Payne's Medium (PM)	In-house preparation	N/A	See recipe below
Antibiotics (e.g.,	Sigma-Aldrich	Various	Prepare stock
rifampicin, ciprofloxacin,			solutions in
chloramphenicol)			suitable solvents
Sterile 96-well microtiter	Corning	3599	Flat-bottom,
plates			tissue-culture
			treated
Sterile 200 µL pipette	Gilson	F171200	Filtered tips
tips			recommended
Sterile Payne's Medium	In-house preparation	N/A	Used for MBC
agar plates			assessment
Dimethyl sulfoxide	Sigma-Aldrich	D8418	For rifampicin stock
(DMSO)			preparation
Sterile distilled water	Thermo Fisher	15230-147	Used for antibiotic
			dilutions

Materials and Reagents Stock Solutions and Reagents

Equipment

Equipment	Brand/Manufacture	Catalogue No.	Notes
Spectrophotometer (OD600)	Thermo Fisher	Genesys 30	Optional for secondary validation
Incubator (42°C)	Memmert	IN55	Essential for archaeal growth
Multichannel pipette	Eppendorf	3123000094	For consistent dispensing
Biosafety cabinet	Thermo Scientific	MSC-Advant age	For aseptic handling

Preparation of Payne's Medium (PM)

Component	Final	Stock Solution
	Concentration	
NaCl	250 g/L	Directly in
		media
MgSO₄·7H₂O	20 g/L	100× stock
KCI	2 g/L	100× stock
CaCl ₂ ·2H ₂ O	0.1 g/L	100× stock
NaHCO₃	0.2 g/L	100× stock
Yeast Extract	5 g/L	Directly in
		media
Casamino	5 g/L	Directly in
acids		media

pH Adjustment: pH 7.2–7.4, autoclave and cool before use.

MIC Protocol

1. Prepare Antibiotic Dilution Series

- 1. Prepare two-fold serial dilutions of antibiotics in Payne's medium.
- 2. Dispense 100 µL of Payne's medium into each well of a sterile 96-well plate.
- 3. Add 100 μ L of antibiotic stock solution to the first column and perform serial two-fold dilutions across the plate.

2. Inoculate the Plate

- 1. Adjust archaeal culture to 1×10⁶ CFU/mL in Payne's medium.
- 2. Add 100 µL of archaeal inoculum to each well (final volume: 200 µL per well).
- 3. Include controls:
 - Positive control: Archaeal culture + no antibiotic
 - Negative control: Medium + no archaeal inoculum

3. Incubation

• Incubate the plate at 42°C for 3 days under aerobic conditions.

4. Determining MIC

• Examine wells for turbidity (visible growth).

• The MIC is the lowest antibiotic concentration with no visible turbidity.

MBC Protocol

1. Subculture from MIC Plate

 Transfer 20 µL from MIC wells that showed no visible growth onto Payne's Medium agar plates.

2. Incubation

- Incubate plates at 42°C for 4 days.
- 3. Determining MBC
 - The MBC is the lowest antibiotic concentration where no colonies grow on agar (indicating ≥99.9% killing).

Example Data

Table 2.1 MIC and MBC determinations of tested antimicrobials against 5×10^5 CFU /mL

of *H. saccharovorum* and *H. volcanii*. All concentrations in µg/mL.

	H. volcanii DS2		H. saccharovorum CSM-52	
Antimicrobial	MIC	MBC	MIC	MBC
Cetylpyridinium chloride	1	1	2	16
Benzalkonium chloride	1	8	1	32
Tetracycline	312	1248	312	1248
Novobiocin	0.06	25	0.02	25
Erythromycin	62.5	250	62.5	250
Rifampicin	15.6	31	15.6	15.6
Bacitracin	156	156	156	156
Chloramphenicol	250	2000	250	2000
Ciprofloxacin	23	23	31.25	31.25
Vancomycin	512	512	512	512
Norfloxacin	19.5	19.5	19.5	19.5
Gentamicin	1024	4096	1024	4096
Neomycin	2500	-	2500	-

Reference:

Fakhoury et al., 2024. Identification and characterisation of two functional antibiotic MATE efflux pumps in the archaeon Halorubrum amylolyticum. *Npj antimicrobials and resistance*. <u>https://www.nature.com/articles/s44259-024-00036-5</u></u>

Matthew, A. et al. NCCLS, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard, M7-A7. (CLSI, Wayne, PA, 2006).