



EUCAST

European Committee
on Antimicrobial
Susceptibility Testing

Public Consultation

EUCAST Reference protocol for MIC determination of anti-mycobacterial agents against isolates of nontuberculous mycobacteria (NTM)

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Please send comments to the EUCAST Scientific Secretary at Mandy.Wootton@wales.nhs.uk by 17 April 2026

Background: *Mycobacterium avium* and *Mycobacterium abscessus* are of the most prevalent nontuberculous mycobacteria (NTM) involved in respiratory and extra-pulmonary human NTM infections. ESCMID, ERS, IDSA and ATS published in 2020 GRADE-based guidelines for the treatment of respiratory diseases due to some NTM including *M. avium*, *M. abscessus*, *M. kansasii* and *M. xenopi* (1). The same group also published systematic review with consensus on treatment for other NTM (2). Consequently, susceptibility testing to macrolides (clarithromycin or azithromycin) and to amikacin was recommended, especially for *M. avium* complex and *M. abscessus* (4). This is why it is important to share a standardized method to determine antimycobacterial agents MIC on NTM, especially for these two species. For the other antibiotics that are often given in combination therapy, and for the other NTMs, the knowledge about MIC distributions and relationship with the outcome is still very much limited. This is why EUCAST-AMST worked on a reference protocol for antimicrobial MIC determination on NTM, in order to expand the data, and prepare future recommendations. As a first attempt, EUCAST-AMST published in 2023 (3) MIC distributions for some of these antimycobacterial agents obtained by using commercial broth microdilution plates, and we realized that we need more robust data.

Clinical use: Before MIC determination is considered for nontuberculous mycobacteria (NTM), either slowly growing (SGM) or rapidly growing NTM (RGM), there is a need to make a thorough species determination. In the protocol below, *M. avium* was taken as a representative of SGM, and *M. abscessus* as a representative of RGM.



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Proposal: The EUCAST reference method for NTM is based on broth microdilution (BMD) using cation-adjusted Mueller-Hinton broth with (SGM) or without (RGM) OADC supplementation.

It follows the principles of the published EUCAST reference protocol for MIC determination of anti-tuberculous agents against isolates of the *Mycobacterium tuberculosis* complex (Version 8.2. January 29th, 2025).

In the annexe document, an example of detailed protocols is given for two NTM species: *Mycobacterium avium*, representative of SGM, and *Mycobacterium abscessus*, representative of RGM, and the main antimycobacterial agents, clarithromycin, azithromycin and amikacin (see § background).

References

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General considerations

Before MIC determination is considered for nontuberculous mycobacteria (NTM), either slowly growing (SGM) or rapidly growing NTM (RGM), **there is a need to make a thorough species determination**. The EUCAST reference method for NTM is based on broth microdilution (BMD) using cation-adjusted Mueller-Hinton broth with (SGM) or without (RGM) OADC supplementation.

The reference protocol consists of two documents:

1. the main document presenting the principles of the EUCAST reference method for MIC determination of anti-mycobacterial agents against isolates of nontuberculous mycobacteria (NTM)
2. an annexe presenting an example of a practical and detailed laboratory protocol

Summary

The EUCAST reference protocol for MIC determination of anti-mycobacterial agents on NTM is a broth microdilution method using:

- a 96-well U-bottom-shaped polystyrene microtiter plate with an untreated surface
- the liquid cation-adjusted Mueller-Hinton broth (CAMHB) made from the broth base with a final concentration of 0.2% glycerol, sterilized. It is enriched with OADC growth supplement for slowly growing mycobacteria (SGM), but not for rapidly growing mycobacteria (RGM)
- a final inoculum of 10^4 - 10^6 CFU/mL of a NTM isolate
- a range of Log_2 concentration values of the anti-tuberculous agent tested, encompassing the targeted MIC value
- at least two growth controls: one with 100% of the inoculum (GC100%) and one with 1% (100-fold dilution, GC1%)
- for water-insoluble agents, a final % of the solvent that is the same in all drug-containing wells and the growth controls wells with no drug, and which does not exceed 1%
- incubation of the microtiter plate at different temperatures, with regard to the mycobacteria species ($36^\circ\text{C} \pm 1^\circ\text{C}$ or $30^\circ\text{C} \pm 1^\circ\text{C}$ in ambient air, no CO_2)
- the MIC value defined as the lowest concentration in mg/L where no visible growth is observed at the time point when both GC100% and GC1% are showing visible growth



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- *M. avium* ATCC 700898 is the quality control (QC) strain for SGM and *M. abscessus* ATCC19977 is the QC for RGM

1. First step: Preparation of broth and antimicrobial agents

- 1.1. A 96-well U-bottom-shaped polystyrene plate, with untreated surface, should be used. Plates or tubes made of polypropylene or other plastic material should not be used. When the plates have been prepared, they should be used as soon as possible (within 12 hours).
- 1.2. For each antimicrobial agent, MIC determination should be done by testing at least eight concentrations in separated wells to cover the full range of potential MIC values.
- 1.3. Prepare cation-adjusted Mueller-Hinton broth (CAMHB) from the base according to the manufacturer's instructions.
 - For SGM, after the medium is autoclaved, allow to cool to room temperature before adding 5% OADC pre-warmed to room temperature (RT; 18-22°C). For some specific species or strains that would require 10% OADC or other supplements (e.g. hemine), this is out of the scope of this protocol.
 - For RGM, most of the isolates do not need addition of OADC in the medium. For some specific isolates, follow the protocol for SGM with OADC.
- 1.4. A stock solution should be prepared for each antimicrobial agent to be tested. This is done by dissolving the active agent in its solvent as recommended in the ISO-20776-1 standard or per recommendation by the manufacturer. As an example, for obtaining a stock solution of 10 240 mg/L, 102.4 mg will be dissolved in 10 mL of the solvent if the potency of the agent is 100%. See examples for the main agents to be tested described in the Tables 1 and 2 of the annexe document. The stock solution is then aliquoted into 0.2 mL/vials and stored at -80°C for a maximum of 12 months, unless otherwise specified by the manufacturer. Thawed vials should not be re-used. Record ordering and batch number of all antimicrobial agents as well as date of stock solution preparation.

For compounds dissolved in water (e.g. amikacin, imipenem, ceftazidime), please follow protocol A from 1.5 to 1.8



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For non-water soluble compounds (e.g. clarithromycin, azithromycin, clofazimine), i.e. which require to use other solvents (e.g. dimethyl-sulfoxide [DMSO]), please follow protocol B from 1.5 to 1.8

Protocol A

- 1.5. Prepare a 4X working solution in two dilution steps in CAMHB or in CAMHB/OADC from an aliquot of a stock solution (as example, see Tables 1 and 2 of the annexe document).
- 1.6. Add 0.1 mL (100µL) CAMHB without or with OADC (see above; for RGM without, for SGM 5% OADC) to all wells, except the peripheral wells, which will be filled by sterile distilled water, as described in Figure 1, in order to prevent desiccation during the incubation time
- 1.7. Add 0.1 mL of the 4X working solution to the wells corresponding to the highest concentration of each agent (C1 in Appendix 1). Do not add any agent to the negative and growth control (GC) wells.
- 1.8. Use a multichannel pipette to make 1:2 dilutions by adding 0.1 mL of the antibiotic solution present in the highest concentration column to the following column and finally discard the last 0.1 mL of the last column. Use the plate outline in Figure 1.

Protocol B

For antimicrobial agents requiring a solvent other than water, the above protocol (A) is not applicable. Since the solvent might have an antimicrobial activity, it is mandatory to keep it at the same minimum concentration in all wells (e.g. 0.5% DMSO). For obtaining this, the working solutions should be diluted separately, and each dilution added one by one in the plate.



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1.5 Prepare the stock solutions in 100% solvent. It should be considering the maximum solubility of the drug, given by the manufacturer. The final concentration of the solvent as well as preparation of the working solutions should be done according to manufacturer's recommendations if present. An example is presented in Table 1 to obtain a final concentration of 0.5% solvent.

The working solutions are prepared in 100% solvent as 200X (final 0.5% solvent) and serial dilutions of the drug should be made in the solvent before its addition to each well.

1.6. Add 10 µL of the 200X solution to 990 µL CAMH broth (including OADC or not according to the species) to prepare 2X working solutions (containing 1 % solvent).

Final solvent concentration should be also tested in the growth control wells (GC100% and GC1%).

1.7. Then add 100 µL of each 2X working solution to the respective drug containing well in the microtiter plate. After the addition of 100 µL of bacterial inoculum the final drug concentrations are 1X with 0.5% solvent.

The proportion of solvent should be the same at each concentration and in each well.

2. Second step: Inoculation of broth, incubation of plates and MIC-determination

2.1. Make sure that broth and plates are at room temperature prior to inoculation. Ensure that the inoculum is prepared from a fresh (within 2 weeks from visible growth for SGM and within one week for RGM) and pure solid culture on any media.

2.2. For SGM, the reference strain *M. avium* ATCC 700898 should be included in each testing round and for RGM the *M. abscessus* ATCC19977.



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The same lot of QC strain should not be used beyond five passages. Briefly, QC strains must be revived from a single-use frozen stock aliquot on solid medium. Passage counting begins with the first subculture after revival. The strain may be subcultured a maximum of five times from this initial culture. After five passages, a new single-use frozen stock aliquot must be thawed and the process restarted.

- 2.3. The colonies should be sampled from several morphologically similar colonies to avoid selecting an atypical variant. If possible, avoid using confluent growth.
- 2.4. Add one 10 μ L loop of colonies in a 10-15 mL sterile screw-cap glass tube containing 5-10 sterile glass beads (3mm). It is important to avoid scraping off medium. Then vortex at least 2 minutes after careful closing of the cap. When clumps are well dispersed, add 5 mL fresh sterile distilled water. Close the cap tightly and homogenize the tube's content by vigorous vortexing until swirling for at least one minute. Wait 30 min for the remaining clumps to settle.
- 2.5. Adjust the turbidity of the supernatant in a new glass tube to McFarland 0.5. The turbidity of the suspension should be determined by using a calibrated nephelometer. If the suspension density is above McF 0.5, add dH₂O until it is reached. If the suspension density is below McF 0.5, it is required to start again from 1.2, otherwise colonies will not be sufficiently dissociated. Vortex for 30 s.
- 2.6. Prepare the final inoculum (10^{-2} dilution of a 0.5 McF suspension) by two steps of dilutions. The volume of bacterial suspension required for one test plate is 10 mL. First, add 1 mL of the 0.5 McF bacterial suspension to 9 mL of culture media and vortex until swirling is obtained for at least 30 seconds. Then, add 1 mL of the 10^{-1} suspension to 9 mL of culture media.
- 2.7. Add 0.1 mL of the final inoculum (10^{-2} dilution of 0.5 McF) to wells containing the antimicrobial agent as outlined in the Appendix 1 starting by the lowest dilution using sterile tips (may be facilitated by using a disposable inoculum reservoir and an 8-channel micropipette).
- 2.8. Growth controls (GC100% and GC1%) should then be inoculated as outlined in the Appendix 1. The GCs consist of



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- a 1:100 dilution of the final inoculum (10^{-2} dilution of 0.5 McF), i.e. 1% of the inoculum present in antibiotic containing wells; GC1%),
- the same inoculum (final inoculum: 10^{-2} dilution of 0.5 McF, i.e. 100% of the inoculum present in antibiotic containing wells; GC100%)
- For antimicrobial agents dissolved in a solvent other than water, GC100% and GC1% should be also added containing the final solvent concentration.

2.9. Check the bacterial quantity in the inoculum by CFU counting on Mueller Hinton agar supplemented (SGM) or not (RGM) with OADC. Plate 10 μ l of the final inoculum (equivalent to 500-5000 CFU, i.e. confluent growth), 10 μ l of 10^{-3} (50-500 CFU) and 10 μ l of 10^{-4} (5-50 CFU) dilutions using calibrated pipette and a spreader. Count the colonies after 14-21 days incubation at $30-36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ depending on species (see below). The target is 1×10^5 CFU/mL with an acceptable range from 5×10^4 CFU/mL to 5×10^5 CFU/mL for a valid test. The results should be recorded.

3. **Third step: incubation and MIC determination**

3.1. After inoculation, cover plates with a plastic lid and then put them in permeable plastic bags or boxes.

3.2. Incubation should be done at ambient air.

3.3. Incubation temperatures are set according to the mycobacterial species

- *M. marinum* and RGM: $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
- MAC, *M. kansasii*, and other SGM: $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
- *M. xenopi*: $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$. If growth is insufficient, testing should be repeated with incubation at $42^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

3.4. Incubation times: Read MIC values as soon as the growth controls GC100% and GC1% are positive.



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- 3.4.1. RGM: Read starting after 3 days incubation until the growth controls GC100% and GC1% are positive. For most RGM, MIC could be read between D3 and D5. If there is not sufficient growth in the GC100% and GC1% at Day 7, repeat the testing. An additional reading after 14 days incubation should be systematically done for macrolides (clarithromycin and azithromycin).
- 3.4.2. SGM: Read starting after 5 days incubation until the growth controls GC100% and GC1% are positive. For most SGM, MIC could be read between D7 and D14. If there is not sufficient growth in the GC100% and GC1% at Day 14, repeat the testing. For some other NTM species, e.g. *M. xenopi* and *M. malmoense*, if poor growth after 14 days, read also at D21 and D28.
- 3.5. Report the MIC value in mg/L according to the lowest antibiotic concentration that inhibits visual growth when the GC100% and GC1% growth controls are positive.



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Table 1. Example of preparation of stock and working solutions of antimicrobial agents (AA) non soluble in water and requiring a specific solvent (e.g. DMSO), obtaining a final concentration of 0.5% of the solvent

Dilution number	Stock solutions stored at -20°C to -70°C	2X working solutions prepared by adding 10 µL of stock solution to 990 µL CAMB (+/- OADC)	Final concentration in plate by transferring 100 µL of the 2X working solutions to plate, to which 100 µL of inoculum are added
	200X	2X	1
	100% solvent	1% solvent	0.5% solvent
1	400 mg/L	4 mg/L	2 mg/L
2	200 mg/L	2 mg/L	1 mg/L
3	100 mg/L	1 mg/L	0.5 mg/L
4	50 mg/L	0.5 mg/L	0.25 mg/L
5	25 mg/L	0.25 mg/L	0.125 mg/L
6	12.5 mg/L	0.125 mg/L	0.06 mg/L
7	6.3 mg/L	0.06 mg/L	0.03 mg/L
8	3.2 mg/L	0.03 mg/L	0.016 mg/L
9	1.6 mg/L	0.016 mg/L	0.008 mg/L
10	0.8 mg/L	0.008 mg/L	0.004 mg/L
11	0.4 mg/L	0.004 mg/L	0.002 mg/L
12	0.2 mg/L	0.002 mg/L	0.001 mg/L
13	0.1 mg/L	0.001 mg/L	0.0005 mg/L



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Figure 1. Outline of the microtiter plate set for MIC determination of one drug and different NTM strains for A) water soluble drugs and B) non–water soluble drugs

A)

	1	2	3	4	5	6	7	8	9	10	11	12
A	200 uL dH2O	200 uL dH2O	200 uL dH2O	200 uL dH2O	200 uL dH2O	200 uL dH2O	200 uL dH2O	200 uL dH2O	200 uL dH2O	200 uL dH2O	200 uL dH2O	200 uL dH2O
	negative	GC100%	Drug	Drug	Drug	Drug	Drug	Drug	Drug	Drug	GC100%	200 uL
B	control	S1	C8	C7	C6	C5	C4	C3	C2	C1	S1	dH2O
	negative	GC1%	Drug	Drug	Drug	Drug	Drug	Drug	Drug	Drug	GC1%	200 uL
C	control	S1	C8	C7	C6	C5	C4	C3	C2	C1	S1	dH2O
	negative	GC100%	Drug	Drug	Drug	Drug	Drug	Drug	Drug	Drug	GC100%	200 uL
D	control	S2	C8	C7	C6	C5	C4	C3	C2	C1	S2	dH2O
	negative	GC 1%	Drug	Drug	Drug	Drug	Drug	Drug	Drug	Drug	GC1%	200 uL
E	control	S2	C8	C7	C6	C5	C4	C3	C2	C1	S2	dH2O
	negative	GC100%	Drug	Drug	Drug	Drug	Drug	Drug	Drug	Drug	GC100%	200 uL
F	control	S3	C8	C7	C6	C5	C4	C3	C2	C1	S3	dH2O
	negative	GC1%	Drug	Drug	Drug	Drug	Drug	Drug	Drug	Drug	GC1%	200ul
G	control	S3	C8	C7	C6	C5	C4	C3	C2	C1	S3	dH2O
	200ul dH2O	200ul dH2O	200ul dH2O	200ul dH2O	200ul dH2O	200ul dH2O	200ul dH2O	200ul dH2O	200ul dH2O	200ul dH2O	200ul dH2O	200ul dH2O

dH2O: sterile distilled water; Negative control: 200 µL medium (CAMHB with [SGM] or without [RGM] OADC

GC100%: 100 µL of medium + 100 µL 10⁻² inoculum suspension; GC1%: 100 µL of medium + 100 µL 10⁻⁴ inoculum suspension

S1-S3; strain 1-3, C1-C8; drug concentration 1-8; C8 is the highest concentration;



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B)

	1	2	3	4	5	6	7	8	9	10	11	12
A	200 uL	200 uL	200 uL	200 uL	200 uL	200 uL	200 uL	200 uL	200 uL	200 uL	200 uL	200 uL
	dH2O	dH2O	dH2O	dH2O	dH2O	dH2O	dH2O	dH2O	dH2O	dH2O	dH2O	dH2O
B	negative	GC100%	Drug	Drug	Drug	Drug	Drug	Drug	Drug	Drug	GC100%	200 uL
	control	S1	C8	C7	C6	C5	C4	C3	C2	C1	solvent f% S1	dH2O
C	negative	GC1%	Drug	Drug	Drug	Drug	Drug	Drug	Drug	Drug	GC1%	200 uL
	control	S1	C8	C7	C6	C5	C4	C3	C2	C1	solvent f% S1	dH2O
D	negative	GC100%	Drug	Drug	Drug	Drug	Drug	Drug	Drug	Drug	GC100%	200 uL
	control	S2	C8	C7	C6	C5	C4	C3	C2	C1	solvent f% S2	dH2O
E	negative	GC 1%	Drug	Drug	Drug	Drug	Drug	Drug	Drug	Drug	GC1%	200 uL
	control	S2	C8	C7	C6	C5	C4	C3	C2	C1	solvent f% S2	dH2O
F	negative	GC100%	Drug	Drug	Drug	Drug	Drug	Drug	Drug	Drug	GC100%	200 uL
	control	S3	C8	C7	C6	C5	C4	C3	C2	C1	solvent f% S3	dH2O
G	negative	GC1%	Drug	Drug	Drug	Drug	Drug	Drug	Drug	Drug	GC1%	200ul
	control	S3	C8	C7	C6	C5	C4	C3	C2	C1	solvent f% S3	dH2O
H	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul
	dH2O	dH2O	dH2O	dH2O	dH2O	dH2O	dH2O	dH2O	dH2O	dH2O	dH2O	dH2O

dH2O: sterile distilled water; Negative control: 200µL of medium (CAMHB with [SGM] or without [RGM] OADC

GC100% with solvent at its final %: 100 µL of medium with solvent final % + 100 µL 10⁻² inoculum suspension; GC1% with solvent at its final %: 100 µL of medium with solvent final %+ 100 µL 10⁻⁴ inoculum suspension

S1-S3; strain 1-3, C1-C8; drug concentration 1-8, C8 is the highest concentration; f is the minimum % of solvent enable growth and avoiding drug precipitation