EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

Reference protocol for MIC determination of anti-tuberculous agents against isolates of the *Mycobacterium tuberculosis* complex in Middlebrook 7H9 broth

Version 7.0. March 1st, 2024

First step: Preparation of broth and anti-tuberculous agents

1. Preparation of culture medium

1.1. A 96-well U-bottom-shaped polystyrene plate with an 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 the same day).

1.2. Prepare Middlebrook 7H9 medium (7H9) from the broth base with a final concentration of 0.2% glycerol, according to the manufacturer's instructions (Tween should not be used). After the medium is autoclaved, allow to cool at room temperature (RT; 18-22°C) and add the OADC at 10% in sterile conditions.

1.3. For each agent, MIC determination should be done by testing at least 8 concentrations in separated wells to cover the full range of potential MIC values (outlined in Tables 1-3 and Figures 1A and B).

2. Preparation of the anti-tuberculous agents

2.1. Protocol A: for water-soluble agents

2.1.1 A stock solution should be prepared as outlined in **Table 1**, by dissolving the active agent in its appropriate solvent as recommended in the ISO-20776-1 standard or if not listed, per recommendation by the manufacturer. As an example, if a stock solution of 10,240 mg/L is needed, 102.4 mg will be dissolved in 10 mL of the solvent if the potency of the agent is 100%. The stock solution is then aliquoted into vials of 0.2 mL and may be stored at -70ºC for a maximum of 12 months unless otherwise is specified by the manufacturer. Thawed vials must not be reused. Record ordering and batch number of all agents as well as date of stock solution preparation.

2.1.2 Prepare a 4X working solution in two dilution steps in 7H9/OADC from an aliquot of a stock solution as outlined in **Table 1** (example for isoniazid and levofloxacin).

2.1.3 Add 100 µL 7H9/OADC to all wells, except the peripheral wells, which will be filled with 200 µL sterile distilled water (dH_2O) in order to prevent desiccation during the incubation time and add 200 µL 7H9/OADC to the negative controls as described in **Figure 1A**.

2.1.4 Add 100 µL of the 4X working solution to the wells corresponding to the highest concentration of each agent (Drug C8 wells in **Figure 1A**). Make sure not to add any agent to the negative and growth control (GC) wells.

2.1.5 Use a multichannel pipette to make 1:2 dilutions by adding 100 µL of the antibiotic solution present in the highest concentration row (Drug C8 wells) to the following row (Drug C7 wells), applying the same procedure throughout the Drug C6-C1 wells and finally discard the last 100 µL of the last row/wells. Use the plate outline in **Figure 1A**.

It should be noted that this step is not adequate for the non-water soluble agents, especially when the solvent should be kept at the same minimum concentration (e.g. a final concentration of ≤1% DMSO in the wells). In this case, the agent working solutions should be diluted separately and then each dilution will be added one by one to the plate. For this, follow the protocol B below.

2.2. Protocol B: for non-water soluble agents (e.g. bedaquiline, delamanid, pretomanid and clofazimine dissolved in DMSO)

General considerations

Drugs should be diluted according to requirements specific for the drug following recommendations from the manufacturer.
The stock solution will be prepared by dissolving the non-water soluble agent in 100% of the adequate and recommended solvent (e.g. DMSO). The working solutions will then be done by dilutions in 7H9 broth-10% OADC (7H9/OADC).
To avoid an inhibitory effect on mycobacterial growth, the final DMSO concentration should be kept at ≤1% in all drug-containing wells and growth control wells in the microtiter plate.
Equally important, the proportion of DMSO should be the same at each drug concentration in the microtiter plate (this differs from the protocol used for water-soluble agents)
In **Tables 2 and 3** examples are shown for the preparation of drug solutions with final concentrations of 0.5% DMSO used for testing bedaquiline, delamanid, pretomanid and clofazimine.

2.2.1 The peripheral wells are filled with 200 µL sterile distilled water (dH2O) in order to prevent desiccation during the incubation time and 200 µL 7H9/OADC to the negative controls as described in **Figure 1B**.

2.2.2 There will be two types of growth controls: GC 100% and GC 1%, both growing in 7H9/OADC with and without the final concentration of the solvent: i.e. 100 µL 7H9/OADC is added to the GC1% and GC100% wells that will not contain the solvent and 100 µL 7H9/OADC with 1% solvent is added to the GC1% and GC100% wells for a final concentration of 0.5% solvent as described in **Figure 1B**.

Drug stock- and working solutions are prepared as follows for a final concentration of 0.5% solvent in the microtiter plate test wells.

2.2.3 Prepare the drug stock solutions as 200X the desired test concentration in 100% solvent and then add 10 µL to 990 µL 7H9/OADC to prepare 2X working solutions (containing 1 % solvent). 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 (See **Table 2**, **Table 3** and **Figure 1B**).

Example: A 10 mL master stock solution of e.g. 400 mg/L can be prepared and diluted as ten two-fold (1:2) stock solutions of 400-0.4 mg/L in 100% DMSO. Each stock solution is then 1:100 diluted in 7H9/OADC (10 µL stock solution + 990 µL 7H9/OADC) to prepare the 2X working solutions of 4-0.004 mg/L (containing 1% DMSO). In the microtiter plate, the final 1X test concentration range of the drug will be 2-0.002 mg/L with a fixed proportion of 0.5% DMSO.

For all the following steps including the MIC reading, biosafety measures recommended for handling cultures of M. tuberculosis including working in safety cabinets must be carefully followed.

3. Preparation and inoculation of the bacterial inocula

3.1. Make sure that broth and plates are at 18-22°C prior to inoculation. Isolates of the *M. tuberculosis* complex to be tested should be grown on solid media (7H10 or 7H11 Middlebrook agar, Löwenstein-Jensen or other egg-based solid media) and sampled from fresh cultures (within 2 weeks after visible colonies/growth). The reference strain *M. tuberculosis* H37Rv ATCC 27294 should be included in each testing round as internal control and the same lot should not be used beyond five subculture passages.

3.2. First add colonies in a 10-15 mL sterile screw-cap glass tube containing 5-10 sterile glass beads (3 mm) then vortex at least 2 minutes after careful closing of the cap. When clumps are well dispersed, add 5 mL fresh sterile dH_2O. Close the cap tightly and homogenize the tube's content by vigorously vortexing the tube to swirling for at least 2 minutes. Wait 30 min for remaining clumps to settle.

3.3. Transfer the supernatant in a new glass tube, vortex for 30 s and then adjust the turbidity to McFarland 0.5 with sterile dH_2O using a calibrated nephelometer. If the suspension density is above McFarland 0.5, add dH_2O until it is reached. If the suspension density is below McFarland 0.5, it is required to start again from 3.2, otherwise colonies will not be sufficiently dissociated. To minimize sedimentation, the bacterial suspension should not be left standing at this point or after any of the subsequent dilutions and has to be well mixed before pipetting onto agar for the colony forming unit (CFU) counts described below.

3.4. Prepare a 1:100 dilution of the bacterial suspension in 7H9/OADC broth by two steps of tenfold dilutions, as described below. The volume of bacterial suspension required for one test plate is 10 mL.

- Prepare first a 10^{-1} suspension by adding 1 mL of the 0.5 McFarland bacterial suspension to 9 mL of 7H9/OADC and vortex until swirling is obtained for at least 30 seconds.
- For the 10^{-2} inoculum, add 1 mL of the 10^{-1} suspension to 9 mL of 7H9/OADC and vortex until swirling is obtained for at least 30 seconds. This 10^{-2} suspension will be used as inoculum for the drug-containing wells and the "100% growth control" (GC100%).

3.5. Additionally, from the 10^{-2} suspension, a 10^{-4} suspension should be prepared by dilution in two steps:
- For the 10^{-3} suspension, add 1 mL of the 10^{-2} suspension to 9 mL of 7H9/OADC and vortex until swirling is obtained for at least 30 seconds.
- For the 10^{-4} suspension, add 1 mL of the 10^{-3} suspension to 9 mL of 7H9/OADC and vortex until swirling is obtained for at least 30 seconds. This 10^{-4} suspension will be used as the inoculum for the "1% growth control" (GC1%).

3.6. Quantify the bacterial inoculum (CFU/mL) by CFU counting on Middlebrook 7H10 agar: using a calibrated pipette, add 10 µL of 10^{-2} (equivalent to 500-5000 CFU, i.e. confluent growth), 10 µL of 10^{-3} (50-500 CFU) and 10 µL of 10^{-4} (5-50 CFU) dilutions, respectively, onto 90 mm agar plates and spread the inoculum evenly over the agar surface. Plates are read after 21 days incubation at 36°C±1°C in ambient air. The target is $1x10^5$ CFU/mL from the 10^{-2} dilution of 0.5 McFarland with an acceptable range from $5x10^4$ to $5x10^5$ CFU/mL for a valid test. The results should be recorded.

3.7. Add 100 µL of the 10^{-2}-diluted inoculum of 0.5 McFarland to the antibiotic containing wells as outlined in the **Figures 1A and 1B** starting by the lowest dilution where 100 µL of antibiotic-dilutions were pre-added earlier using sterile tips. The procedure may be facilitated by using a disposable inoculum reservoir and an 8-channel micropipette with the outer channels (corresponding to dH_2O wells) removed.

3.8. Growth controls (GC100% and GC1%) should then be inoculated as outlined in the **Figures 1A and 1B**.
- For each strain tested in the microtiter plate with water-soluble agents, there are two GC100% and two GC1% to which 100 µL of 7H9/OADC and thereafter 100 µL of the bacterial inoculums are added (**Figure 1A**).
- For each strain tested in the microtiter plate with the non water-soluble solvent, there are one GC100% with and one without the solvent, as well as one GC1% with and one without the solvent (**Figure 1B**).
  Add 100 µL of 7H9/OADC to the GCs without solvent and add correspondingly 100 µL of 7H9/OADC containing 1% solvent (see plate outline in **Figure 1B**). When 100 µL of the bacterial inoculums are added, the final solvent proportion in the GCs is 0.5%.

Important note: Due to the limited dissolution of the non-water soluble drugs, the working solutions in 7H9/OADC should be added without delay to the microtiter plate, mixed with the bacterial inoculum and immediately incubated.

4. Incubation of plates and MIC determination

4.1. After inoculation, cover plates with a plastic lid and then put them in plastic bags or boxes, as used in the mycobacteriology laboratory, and incubate at 36°C±1°C in ambient air. A maximum of 3 microtiter plates can be stored on top of each other. Agar plates for inoculum counting are incubated too at 36°C±1°C in ambient air. To avoid dryness of the agar and the microtiter plates, it is possible to add sterile water in the reservoir of the box or use another way to prevent desiccation.

4.2. The plates are read using an inverted mirror or an automated reading equipment first after 7 days of incubation, then at 10 days and 14 days of incubation (visual growth of GC100% AND the GC1% is mandatory for the MIC reading and most often the GC1% is positive after 10 or 14 days of incubation).

- The negative control must show no growth for the test to be valid.
- If the GC100% is positive, check the GC1%.
- If the GC1% also shows visible growth (usually weaker positivity than GC100%), the MIC is determined.
- If there is still no visual growth of the GC1% after 14 days, incubate until a maximum of 21 days.

4.3. The growth on the agar plates is checked at day 21 and if negative or below the expected count, the test is repeated with a new fresh inoculum.

4.4. The MIC corresponds to the lowest concentration of the agent where no visible growth is observed when the GC100% and GC1% show visible growth.
Report the MIC value in mg/L. The day of reading the MIC should be recorded.

Table 1. Examples of preparation of anti-tuberculous agents that were evaluated using the reference protocol by EUCAST-AMST against *M. tuberculosis* H37Rv ATCC 27294.

Antimicrobial	Sigma No	Solvent	Stock conc** (mg/L)	Dilution:1 (7H9)***	Dilution:2 (7H9/OADC)	Working conc. (4X) in 7H9/OADC (mg/L) 1 mL=10 plates	Final concentration in 7H9 broth (mg/L)
Isoniazid	I3377	dH_2O	10 240	1:64	1:40	4	1-0.008
Levofloxacin	28266	*	10 240	1:64	1:10	16	4-0.03

*Add powder to 50% dH_2O of the total volume and then 1 mol/L NaOH dropwise to dissolve. Then add dH_2O to the final volume.

****Calculate the amount of drug to dissolve in 10 mL according to potency: m = V*p/P. m=mass of the antimicrobial agent (powder) in g; p=concentration of the stock solution in mg/L; P=potency of the antimicrobial agent (powder) in mg/g (ie 67% potency means 670mg/g or and 100% potency 1000 mg/g); V=volume of diluent in Liter.

***The addition of OADC is not necessary in this step as it is for further dilution only.

Table 2. Preparation of non-water soluble anti-tuberculous agents that were evaluated against *M. tuberculosis* H37Rv ATCC 27294 using the reference protocol by EUCAST-AMST.

Antimicrobial	Supplier/lot number	Solvent	200X stock solutions (mg/L) prepared in 100% DMSO	2X working solutions (mg/L) prepared by adding 10 µL of stock solution to 990 µL 7H9/OADC, 1 mL ~ 10 plates	Final concentration (mg/L) in 7H9 media
Delamanid	Otsuka S16K90	DMSO	12.5-0.1	0.12-0.001	0.06-0.0005
Bedaquiline	Janssen JNJB60025252	DMSO	200-1.6	2-0.016	1-0.008
Pretomanid	TB Alliance 20121393	DMSO	400-3.2	4-0.03	2-0.016
Clofazimine	Sigma SLBV7150	DMSO	400-3.2	4-0.03	2-0.016

Table 3. Example of preparation of stock and working solutions of antimicrobial agents 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 7H9/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	1x
	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

Figure 1A. Outline of the microtiter plate set for MIC determination of water soluble drugs

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

dH2O: sterile distilled water; Negative control: 200 µL 7H9/OADC
GC100%: 100 µL of 7H9/OADC + 100 µL 10^{-2} inoculum suspension**; GC1%**: 100 µL of 7H9/OADC+ 100 µL 10^{-4} inoculum suspension
S1-S3: strain 1-3, **C1-C8**: drug concentration 1-8

Figure 1B. Outline of the microtiter plate set for MIC determination of non–water soluble drugs.

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

dH2O: sterile distilled water; Negative control: 200 µL 7H9/OADC
GC100%: 100 µL of 7H9/OADC+ 100 µL 10^{-2} inoculum suspension**; GC1%**: 100 µL of 7H9/OADC+ 100 µL 10^{-4} inoculum suspension
GC100% + solvent: 100 µL of 7H9/OADC with 1 % solvent + 100 µL 10^{-2} inoculum suspension; **GC1% + solvent**: 100 µL of 7H9/OADC with 1% solvent + 100 µL 10^{-4} inoculum suspension
S1-S3: strain 1-3, **C1-C8**: drug concentration 1-8