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


Appendix 1. Plate outline (7H9 AMST In-house).

1. First step: Preparation of broth and anti-tuberculous agents:
   1.1. A 96-well U-bottom-shaped polystyrene plate 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. 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 Table 1 and Appendix 1).
   1.3. Prepare Middlebrook 7H9 medium (7H9) from the base, according to the manufacturer’s instructions. After the medium is autoclaved, allow to cool to 50°C in a pre-warmed water bath before adding 10% OADC which should be pre-warmed to room temperature (RT; 18-22°C). For each 96-well plate, 10 mL of ready-made 7H9/OADC broth is needed.
   1.4. A stock solution should be prepared as outlined in Table 1, by dissolving the active agent in its 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 0.2 mL/vials and may be stored at -80°C for a maximum of 12 months unless otherwise specified by the manufacturer. Thawed vials should not be reused. Record ordering and batch number of all agents as well as date of stock solution preparation.
   1.5. 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, levofloxacin and amikacin).
   1.6. Add 0.1 mL 7H9/OADC to all wells, except the peripheral wells, which will be filled in by sterile distilled water, as described in Appendix 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). Make sure not to 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 row to the following row and finally discard the last 0.1 mL of the last row/wells. Use the plate outline in Appendix 1. It should be noted that this step is not adequate for some agents, especially when the solvent should be kept at the same minimum concentration (e.g. 1% DMSO). In this case, the agent working solutions should be diluted separately and then each dilution added one by one.
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.

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

2.1. 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, LJ or other egg-based solid media) and sampled from fresh cultures (within 2 weeks from visible growth). The reference strain *M. tuberculosis* H37Rv ATCC 27294 should be included in each testing round and the same lot should not be used beyond five passages.

2.2. Bacterial colonies should be sampled from several morphologically similar colonies (when possible to avoid selecting an atypical variant) at approximate 1 mg (4 loops of 1µl or a full 3 mm loop). Emulsify the colonies along the inside wall of the tube using an applicator stick or plastic loop. It is important to avoid scraping off medium.

2.3. First add colonies in a 10-15 mL sterile screw-cap glass tube containing 5-10 sterile glass beads (3mm) 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 vigorously vortexing the tube to swirl for at least 2 minutes. Wait 30 min for remaining clumps to settle.

2.4. Adjust the turbidity of the supernatant in a new glass to McFarland 0.5 by sterile dH2O. Vortex for 30 s. 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 2.4, otherwise colonies will not be sufficiently dissociated. The turbidity of the suspension should be determined by using a densitometer (suspension turbidity meter).

2.5. Prepare a 1:100 dilution of the bacterial suspension in 7H9/OADC broth by two steps of tenfold dilutions. The volume of bacterial suspension required for one test plate is 10 mL. Prepare a 10⁻¹ suspension by adding 1 mL of the 0.5 McF 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.

2.6. Additionally, from the 10⁻² suspension (growth control (GC100%), a 10⁻⁴ (GC1%) suspension should be prepared in two dilution steps: 1+9 mL (10⁻³), then 1+9 mL (10⁻⁴). This will be used as a GC for checking the inoculum and to assess the MIC values.

2.7. Check the bacterial quantity in the inoculum by CFU counting on Middlebrook 7H10 agar: plate 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 and read after 14-21 days incubation at 36°C±1°C. The results should be recorded and the target is 1x10⁵ CFU/mL from the 10⁻² dilution of 0.5 McF with an acceptable range from 5x10⁴ to 5x10⁵ CFU/mL for a valid test. The results should be recorded.

2.8. Add 0.1 mL of the 10⁻² inoculum of 0.5 McF to antibiotic containing wells as outlined in the Appendix 1 starting by the lowest dilution where 0.1 mL of antibiotic-dilutions were pre-added earlier using sterile tips (may be facilitated by using a disposable inoculum reservoir and an 8-channel micropipette with the outer channels corresponding to dH2O removed).

2.9. Growth controls (GC100% and GC1%) should then be inoculated as outlined in the Appendix 1. The GCs consist of a 1:100 dilution of the 10⁻² inoculum of 0.5 McF (i.e. 1% of the inoculum present in antibiotic containing wells; GC1%), and the same inoculum (10⁻² suspension of 0.5 McF (i.e. 100% of the inoculum present in antibiotic containing wells; GC100%).
3. **Third step: incubation and MIC determination**

3.1. After inoculation, cover plates with a plastic lid and then put them in O₂-/CO₂-permeable plastic bags or boxes and incubate at 36°C±1°C. A maximum of 3 plates can be stored on top of each other.

3.2. Read the plates using an inverted mirror, first after 7 days of incubation and then at 14 days of incubation (visual growth of the GC1% is mandatory for reading and more often present at the day 14 reading). The negative control should 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%), MIC can be determined as the lowest concentration of the agent where no visible growth is observed. If there is still insufficient growth of the GC1% after 14 days, incubate until a maximum of 21 days.

3.3. Report the MIC value in mg/L

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### 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

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Sigma No</th>
<th>Solvent</th>
<th>Stock conc**</th>
<th>Dilution:1 (7H9)***</th>
<th>Dilution:2 (7H9/OADC)</th>
<th>Working conc. in 7H9/OADC (mg/L) 1mL=10 plates</th>
<th>Final concentration in 7H9 broth (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>I3377</td>
<td>dH₂O</td>
<td>10 240</td>
<td>1:64</td>
<td>1:40</td>
<td>4</td>
<td>1-0.008</td>
</tr>
<tr>
<td>Amikacin</td>
<td>A1774</td>
<td>dH₂O</td>
<td>10 240</td>
<td>1:64</td>
<td>1:5</td>
<td>32</td>
<td>8-0.06</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>28266</td>
<td>*</td>
<td>10 240</td>
<td>1:64</td>
<td>1:10</td>
<td>16</td>
<td>4-0.03</td>
</tr>
</tbody>
</table>

*Add powder to 50% dH₂O of the total volume and then 1 mol/L NaOH dropwise to dissolve. Then add dH₂O 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.