**EUCAST form for General Consultation**

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

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<tr>
<th>Comment from (name, contact details)</th>
<th>Comments</th>
<th>EUCAST Responses</th>
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<tr>
<td>Michaela Jonsson Nordvall</td>
<td>The protocol don’t specifically say that the reference strain should be analyzed with every clinical strain. Should this be specified?</td>
<td>The reference strain <em>M. tuberculosis</em> 27294 should be used as a QC-strain when clinical isolates are analyzed. This will be specified in the calibration SOP. <strong>Action:</strong> The protocol was clarified in 2.2 according to the suggestion.</td>
</tr>
<tr>
<td>Clinical microbiology TB-lab</td>
<td>2.2 “Bacterial colonies should be sampled from several morphologically similar colonies (when possible to avoid selecting an atypical variant)” This could be difficult to do and if there is obvious growth of different looking colonies variants my suggestion is to re-cultivate the colonies of interest in a new tube before analyzing MIC.</td>
<td>We agree that it should always be assured that a pure culture is used for MIC testing in the reference method. On the other hand, it should be avoided to passage the isolates too many times from the primary culture, otherwise, the isolate may start to lose its characteristics. Therefore, it is necessary to pick several colonies to get a representative sample of the population for testing. This is also specified in the same way in the latest ISO-recommendation for rapidly growing bacteria.</td>
</tr>
<tr>
<td>University Hospital 581 85 Linköping</td>
<td></td>
<td></td>
</tr>
<tr>
<td><a href="mailto:michaela.jonsson.nordvall@regionostergotland.se">michaela.jonsson.nordvall@regionostergotland.se</a></td>
<td></td>
<td></td>
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<tr>
<td>19th of May 2019</td>
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<td>Action: No change required.</td>
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<tr>
<th>2.3 Vortexing (2 min is a long time) a glass tube should be avoided to minimize the risk that it cracks. Also the risk that the vortex leaves marks on the tube generating misleading OD measurement. My suggestion is to use a polystyrene tube when vortexing and then transfer the suspension to glass tube for OD-measurement.</th>
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| The common experience from decades of testing in the AMST laboratories are that the glass tubes, or Erlen flasks, will not crack if handled properly. Additionally, a polystyrene tube can change diffusion of light when determining the inoculum turbidity, which would need further validation. As using a polystyrene tube may be preferred by some laboratories, this will be considered for further investigation but will not warrant a change to the protocol at this stage. |

| Action: No change required. |

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<tr>
<th>I understand, that EUCAST intends to have a non-commercial method for TB-DST. But what do you exactly mean with your statement: ‘When other methods are to be used in clinical trials and routine susceptibility testing for the MTBC, it is necessary to properly calibrate and validate these against the reference method.’ ???</th>
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| In case of MGIT for routine DST, thousands of results have been published and much more performed. Do you also intend to include regular DST performed this method into your statement? If not, could you please clarify this. |

| In accordance to susceptibility testing done for all other bacteria and fungi within EUCAST, a reference method is needed to enable the use of EUCAST clinical breakpoints. For this reason, other methods, which are routinely used in the clinical microbiology laboratories, need to be calibrated against the reference method. DST by MGIT is one of these methods used for the MTBC but needs to be calibrated to the |

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Dr. Elvira Richter  
Labor Limbach, Heidelberg  
Elvira.richter@labor-limbach.de  
3rd of June 2019
### EUCAST form for General Consultation

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<th>Action</th>
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<tr>
<td>No change required.</td>
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<td>References 1 and 2 presumably have been reversed.</td>
<td>The references have been rechecked and are cited in the correct order.</td>
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<td>Within the demonstration of the test results I cannot get the difference between 'visual reading' and '1:100 reading'.</td>
<td>Visual reading means the traditional way of estimating MIC (lack of visual growth) whereas the 1:100 reading means reading compared to a 1:100 diluted control (i.e. refers to the indirect proportion method). In the reference method, visual growth in the 1:100 diluted control is required to read the MIC, which is done visually and without comparing to the 1:100 control. Visual reading in liquid media is more subjective than when it is done according to colony counting in the Middlebrook 7H10 method. However, we still prefer the 1:100 diluted control as a cut off for when the MIC reading should be done, because we observed that it results in a more accurate MIC determination, as it will control for the inoculum density. This is particularly important in <em>M.</em></td>
</tr>
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</table>
| Lynette Berkeley, PhD, M.T. (ASCP), Lynette.berkeley@fda.hhs.gov | Timely. The rationale is realistic. The protocol is logically written, and it is clear, the concepts are explained, and examples are provided when necessary. | **tuberculosis** isolates when rough colonies are not well dissociated.  
**Action:** No change required. |
|---|---|---|
| Koné Kaniga kkaniga@its.jnj.com | Result summary –reference method. 10th of May 2019 | Thank you for the positive feedback.  
**Action:** No response needed. |
| | Page 10: inoculum control: The overall mean (target inoculum size) of $1 \times 10^5$ CFU/ml is skewed for the following reasons: | The result summary is not subject to general consultation but input is appreciated.  
The reference method was not set only on the data presented but these data showed the stability of the method in the four AMST laboratories. In the future when the reference method will be applied to more isolates and anti-tuberculous agents, some of the points mentioned will be reconsidered but not necessarily revised. However, the reference protocol will remain as it is, until sufficient data will be collected and breakpoints set for each anti-tuberculous agent. |
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<td>1.</td>
<td>The number of participating laboratories in the study was sub-optimum because it does not allow the exclusion of the least concordant lab and redo a sensitivity analysis to assess the impact of that lab on the mean result.</td>
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<tr>
<td>2.</td>
<td>Lab 2 contributed 3.2 to 4 time more data (that was lower value of all) than other Labs. If one were to exclude Lab 2 the mean would be 3 x 10^5 CFU/ml which is within 0.5-log of the target inoculum size of 5 x 10^5 CFU/ml using McFarland 1 diluted 50-fold in an 8-laboratory study generating &gt;200 datapoints (Kaniga et al. J Clin Microbiol 54:2956 –2962). Based on this observation, it would appear that only Lab 2 that contributed more data than all other labs combined (16/30) had in fact reached the &quot;logical&quot; target inoculum of 6 x 10^4 CFU/ml based upon McFarland 0.5 diluted 100-fold.</td>
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**Action:** No change required.

1. Since all data is within the preset target at +/- one MIC dilution for all sites >95% of observations, we do not see the point in making a sensitivity analysis as it will not change the conclusions.

**Action:** No change required.

2. Even if one laboratory contributed with more data, we see no reason to exclude any laboratory as long as there is a good agreement with the QC-strain as well as the inoculum. What is the logical target inoculum varies between studies and guidelines. In fact, most approaches (CLSI, ISO and Thermofisher) do not use a 1 McF inoculum but 0.5 McF which is then further diluted.

**Action:** No change required.
| 3. | The results from each lab assumes that the same inoculum was obtained every day of testing which is impossible. |
| 4. | If the inoculum size shown for each lab is in fact an average of all testing dates at that lab, an analysis of inoculum distribution is needed to identify outliers. As indicated in point 1, the exclusion of the least concordant data should be done and redo a sensitivity analysis to assess the impact of that those data on the mean result. |
| 3-4. | The same inoculum was not obtained every day. The inoculum reported are means for each laboratory and were estimated based on CFUs. A detailed CFU count list for each lab is beyond scope for the result summary but can be shared upon request. As results are +/- one MIC dilution within the pre-set target, a sensitivity analysis based on the inoculum will not change the conclusions. |

**Action:** No change required.

**Page 15: EUCAST AMST data (Mtb H37Rv, ATCC 27294):**

The choice of H37Rv is relevant but does not represent the real-life situation where laboratories test clinical isolates of MTBC e.g., MDR-TB including pre- and XDR-TB. These organisms grow more slowly than pan-susceptible strains. For those organisms, the inoculum size and incubation time matter. A formal EQA study with an EQA panel that includes clinical isolates some with well characterized resistance phenotype would have been optimum.

**Action:** The result summary is not subject to general consultation but input is appreciated. We agree that EQA studies including M/XDR isolates are important to study at later stages when the reference method has been launched and EUCAST clinical breakpoints for that method are set. Without a stable reference method for susceptible isolates, it is very difficult to
perform further testing as there is no reference to validate the results.

To enable the testing of resistant isolates, there will be a need to assure that there is a suitable and stable method for susceptible isolates first, including QC-ranges for QC-isolates. The EUCAST strategy is rather to establish wild-type distributions and stability of the method in susceptible isolates before defining breakpoints and performing external control assurance programmes or panels.

**Action:** No change required.

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**Koné Kaniga**  
kkaniga@its.jnj.com

**Page 17: MIC determination – Overview – Last bullet:**

Since the MIC well is not plated there is no way to assess 99% inhibition of growth. However, the definition in the reference method Step 3.2 "MIC = no visible growth" is more realistic

The result summary is not subject to general consultation but input is appreciated.

In the reference method, visual growth in the 1.100 diluted control is required to read the MIC (which is done visually and without comparing to the 1:100 control). Visual reading in liquid media is more subjective.
than when it is done according to colony counting in the Middlebrook 7H10 method. However, we still prefer to refer to the 1:100 diluted control as a cut off for when the MIC reading should be done, because we observed that it results in a more accurate MIC determination and controlled inoculum.

**Action:** No change required.

### Plate map:

**Koné Kaniga**  
[kkaniga@its.jnj.com](mailto:kkaniga@its.jnj.com)

The current plate layout of having the GC 100% near the negative control (rows C, D and E) represents a risk of contamination of the negative control wells.

Instead, it might be more prudent to set all the GC 1% (low density bacterial load) between the negative control and the highest concentration of the drugs which might not show growth for most drugs.

Conversely, the GC 100% should be set between dH₂O and the lowest concentration of the drug which should show growth for all drugs. Please refer to enclosed Excel spreadsheet

We find it suitable to make sure that there is in particular no contamination even from the GC100% well.

To ensure the comparison to both GC100% and GC1% and to adequately control for contamination we prefer to keep the current plate outline.

To ensure the comparison to both GC100% and GC1% and to adequately control for contamination we prefer to keep the current plate outline.

**Action:** No change required.
| Koné Kaniga  
| kkaniga@its.jnj.com |
| Coordinates Reference protocol for MIC determination |
| **Page 1 Step 1.8:**  
| Laboratories will most likely not prepare plates each time they are going to perform AST. Please indicate whether the plates could be frozen (and for how long) and use another day  
| The current version of the reference method is adapted for freshly made plates.  
| The applicability of the method is an important aspect but it is necessary to validate the performance of any deviation from the reference method such as the use of frozen plates before it is implemented.  
| **Action:** It was specified in the protocol that plates should be used when they are prepared. The possibility to use frozen plates or with freeze-dried formulations will most likely be addressed in future calibration studies of surrogate MIC methods. |
| Koné Kaniga  
| kkaniga@its.jnj.com |
| **Page 3 Step 3.2:**  
| Unlike MGIT where growth/no growth of drug-containing tubes is called when the drug-free  
| We agree with the comments but the reference method would usually not be the standard |
control has flagged based upon a standardized growth unit, calling the MIC for XDR-TB isolates that grow much slower than pansusceptible strain based on visual growth status of the control at 1% inoculum might lead to two things:

1. A large number of pre-XDR and XDR-TB isolates would not have achieved sufficient growth (of 100 organisms) in the control well even at Day-14.

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1. If necessary, the incubation can be prolonged until the 1:100 control is positive as presented in the protocol. Based on a previous publication (Rancoita PM. et al AAC 2017) and the CLSI guidelines, we have now added an additional reading time point at 21 days if there is not enough growth of the 1:100 diluted control at day 14. On an additional note, it may be possible to use other methods (MGIT and others) calibrated against the reference method if it
2. A significant number of slower growing clinical isolates might be assessed as false-susceptible (even if growth was observed in the control well). This in turn may lead to high microbiologic failures if those drugs are left in the treatment regimen.

| | | will not work for particular strains, as long as it is calibrated to the reference method. Moreover, it is not only highly resistant strains that may show slow growth but also some susceptible and monoresistant isolates.  
**Action:** The protocol was clarified as described above in 3.2.  
2. We are aware that, as for all the methods, long incubation periods may affect test quality. This is one reason why the breakpoints will be defined on the basis of susceptible strains. For further validation in the reference method, some MDR/XDR strains could be tested but also susceptible isolates with a longer time to grow than other isolates (e.g. other species of *M. tuberculosis* complex).  
**Action:** No change required. |
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**My general conclusion:**
The reference protocol for MIC determination is well written and in a concise manner. The only notable difference in comparison to Kaniga et al. (J Clin Microbiol 54:2956–2962) is the inoculum preparation. In the former, a 0.5 McFarland is diluted 100-fold targeting a final inoculum of $1 \times 10^5$ CFU/ml (acceptable range $5 \times 10^4$ to $5 \times 10^5$ CFU/ml). In the latter, a 1 McFarland is diluted 50-fold targeting a final inoculum of $5 \times 10^5$ CFU/ml ± 0.5-log (acceptable range $1.8 \times 10^5$ to $8.1 \times 10^5$ CFU/ml). Thus, there is a significant overlap between the inoculum range for the two methods. Unlike β-lactam antibiotics, bactericidal drugs such as bedaquiline are not impacted by the inoculum size within 0.5-log of the target inoculum. The calibration study would help clinch these subtle differences and ultimately any impact on breakpoints.

**Action:** No change required.

Both the CLSI and the ISO guidelines recommend 0.5 McF, which is diluted 1:100. The CLSI accepts a range from $4 \times 10^4$ to $1 \times 10^6$ for mycobacteria whereas ISO (rapidly growing bacteria) recommends $2-8 \times 10^5$ CFU/ml and that 50 ul of a 1:100 diluted control should be mixed with 50ul of an antimicrobial solution. Estimating exact numbers of CFUs of the *M. tuberculosis* complex is challenging due to clumping, as are estimations of McF suspensions. We note that there are also other differences between the methods like the inclusion of Tween or not, and the preparation of the inoculum. As commented, any differences between the methods will have to be further assessed by a calibration using the EUCAST calibration SOP for mycobacteria. The inoculum is clearly an important issue. We developed the reference method to be appropriate for most anti-tuberculous agents.
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| Frederick A Sirgel  
fas@sun.ac.za  
Stellenbosch University,  
Cape Town, South Africa.  
30th of May 2019 | 1. Table 1 Column 5 indicates that the 1:64 dilution of the drugs is done in 7H9 medium lacking OADC. Should OADC be added or is it excluded to prevent protein binding?  
This issue has been discussed and because the media is used for dilution only and not growth, we have chosen to exclude OADC in this step.  
**Action:** The omission of OADC in this dilution step has now been specified in the footnote to table 1. |  
| | 2. Drug stock solutions are suggested to be stored at -80°C for 12 months – should it not be 6 months?  
This is a valid point, as there may be variations for different drugs. For drugs where there are instructions in the ISO-guidelines, we adhered to those guidelines. For other drugs, it has been specified that the drugs should be stored according to the manufacturer.  
**Action:** The protocol has been clarified in 1.4 |  
| | 3. Can the six 100% GCs be placed in the same row (B2 to G2) and the 1% GC in row 11 (B2 to G2) – see appendix?  
We prefer to have both controls in the same row as it facilitates the reading and the comparison of growth.  
**Action:** No change required. |  
| | 4. I have rearranged the protocol (see below) in a way that makes it easier for me to follow. Some suggestions may be useful to consider.  
We agree that rearrangements of the protocol may make it easier to follow but we would like to keep the protocol in this format as it followed a standard format. If there is a need for a personal rearrangement with |
the same content, that is a possibility. The common experience from decades of testing the AMST laboratories are that the glass tubes will not crack if handled properly. Additionally, a polystyrene tube can change diffusion of light when determining the inoculum, which would need further validation. As using a polystyrene tube may be preferred by some laboratories, this will be considered for further investigation and validation but will not warrant a change to the protocol at this stage. Zip lock bags as suggested may be a good alternative to other plastic bags.

**Action:** No change required.

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<th>Comment from (name, contact details)</th>
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<th>EUCAST Responses</th>
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<tbody>
<tr>
<td>Alexandra Aubry, <a href="mailto:alexandra.aubry@sorbonne-universite.fr">alexandra.aubry@sorbonne-universite.fr</a></td>
<td>Many thanks for proposing reference method for the determination of MICs in Mycobacterium tuberculosis complex.</td>
<td>Thank you for the positive feedback. <strong>Action:</strong> No response needed.</td>
</tr>
<tr>
<td>Nicolas Veziris, <a href="mailto:nicolas.veziris@sorbonne-universite.fr">nicolas.veziris@sorbonne-universite.fr</a></td>
<td>There are clear benefits in using broth microdilution in 7H9. However, considering broth microdilution as the single reference method may have the following drawbacks:</td>
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<td>Jérôme Robert, <a href="mailto:jerome.robert@sorbonne-universite.fr">jerome.robert@sorbonne-universite.fr</a></td>
<td>- the decrease of concordance between 7H9 and 7H10 medium while the incubation time increases is a matter of concern given the slow growth of several clinical isolates in liquid media, especially MDR and XDR isolates or <em>M. africanum</em> and <em>M. bovis.</em></td>
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<td>- the safety issue should be considered since liquid culture in 96-well U-bottom-shaped plates covered by a removable lid could expose to aerosol or projection biohazards in case of leakage (in contrast with solid media)</td>
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<td>We agree with the comments but the reference method would most likely not be the standard method used for testing MDR/XDR isolates in diagnostic laboratories. It may be possible to use other methods (MGIT and others) if the reference method will not work for particular strains, as long as the surrogate method is calibrated to the reference method. Moreover, it is not only highly resistant strains that may show slow growth but also some susceptible and monoresistant isolates.</td>
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<td>Action: No change required.</td>
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<td>Biosafety is an issue with any method that is used for MTBC. As long as there are adequate biosafety precautions, both liquid and solid media could be used safely which also includes microtiter plates with removable lids. The use of removable lids is regarded not to increase biohazard risks compared to the plastic lid but rather the reverse since if removable lids are</td>
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<td>Nancy Wengenack, Mayo Clinic and CLSI M24/M62 group; <a href="mailto:wengn@mayo.edu">wengn@mayo.edu</a> 21th of June 2019</td>
<td>I very much like the proposal to use BMD/MIC determination as the reference standard for <em>M. tuberculosis</em> complex (MTBC) AST and congratulate EUCAST for taking on this task. I have two comments/questions just for clarity: • does the new proposal require groups to re-verify existing methods already in use by clinical labs (eg., MGIT, agar proportion) against the BMD/MIC method? This would add significant burden for these labs and I’m not sure it would add value. Perhaps a EUCAST (or EUCAST/CLSI?) multicenter study could do</td>
<td>Thank you for the positive feedback. <strong>Action:</strong> No response needed. 1. There will be a need to calibrate other methods (such as MGIT) against the EUCAST reference method to use EUCAST breakpoints but that is clearly beyond scope for routine laboratories. We compared</td>
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<td>this so individual labs do not have to take on this task?</td>
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<td>the reference method to the agar proportion method in the AMST study but this will need be further investigation. The suggestion for a multicentre study including EUCAST, CLSI and other stakeholders would be an excellent approach. EUCAST will publish a calibration SOP for that purpose along with the final version of the reference method.</td>
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<td>I was confused about why only INH, amikacin, and levofloxacin were listed in the proposal. I’m assuming that BMD/MIC is proposed as the reference method for all current drugs and for future new drugs tested against MTBC? If this is correct, it would be helpful to state this clearly in the document and add preparation instructions for other agents to Table 1 in the protocol.</td>
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<td>2. The EUCAST reference method is suggested for all current drugs against MTBC. Isoniazid, amikacin and levofloxacin are included just as examples. The reference protocol can be used for any drug as described in 1.4 but we expect to add more detailed preparation instructions for other drugs in future versions of the reference method to facilitate further testing.</td>
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<td>Action: No change required in the present version of the reference method.</td>
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<td>Philip Fowler 25th of June <a href="mailto:Philip.fowler@ndm.ox.ac.uk">Philip.fowler@ndm.ox.ac.uk</a></td>
<td>The proposed protocol only uses the standard H37rV TB strain, which belongs to Lineage 4. There is now some evidence appearing that the mode of wild-type distributions for several drugs (e.g. fluoroquinolones and rifamycins) depends on lineage. Hence one can calibrate secondary methods to the reference method using H37rV, but if this is true, one should not determine clinical breakpoints based on ECOFFs purely using H37rV. A second reference strain belonging to Lineage 2, at a minimum, should be added. There is a risk the method will give a highly-accurate wrong breakpoint.</td>
<td>We agree that several control strains may be used in the future and that strains of different lineages should be tested. However, the reference method is purely a protocol for susceptibility testing. A guideline on how to set ECOFFs is detailed in a separate EUCAST SOP. Certainly, setting ECOFFs will require clinical isolates representing all lineages (1-7) in at least 100 wild-type isolates from at least 5 separate laboratories. Thus, the ECOFF would not be set only on the standard H37Rv strain (ATCC 27294) and thus there is no risk of a highly accurate, wrong breakpoint. While setting ECOFFs, the role of the H37Rv ATCC 27294 is more of an internal QC strain. There are some early data suggesting that there are lineage specific effects on wild type MIC distributions for some drugs against...</td>
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<tr>
<td>Philip Fowler 25th of June <a href="mailto:Philip.fowler@ndm.ox.ac.uk">Philip.fowler@ndm.ox.ac.uk</a></td>
<td>What exactly are the criteria for calibrating a secondary method? Will these also be subject to a consultation?</td>
<td>The calibration SOP has been developed within EUCAST and will be published along with the final version of the reference method. <strong>Action:</strong> No change required.</td>
</tr>
<tr>
<td>NWGA, Christoffer Lindemann, <a href="mailto:pc-linde@online.no">pc-linde@online.no</a></td>
<td>The proposed reference method for AMST is supported by NWGA. We congratulate the AMST subcommittee for their great work and effort establishing this method.</td>
<td>Thank you for the positive feedback. <strong>Action:</strong> No response needed.</td>
</tr>
<tr>
<td>Esther X. Perez-Herran <a href="mailto:esther.x.perez-herran@gsk.com">esther.x.perez-herran@gsk.com</a> 26th of June 2019</td>
<td>The inoculum determination and plate read out are visual, so standardization is quite challenging. We realize there may be practical limitations, but some sort of quantifiable method would be desirable.</td>
<td>1. We agree that it would have been better to present a quantifiable method similar to the colony count in Middlebrook 7H10 but solid media preparation, inoculation and storage were regarded as a limiting factor for</td>
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</table>
| Have you thought about compounds which requires a non-standard conditions to show activity (Ej: non-standard carbon source, pH, non-replicating, cmpds with only intracellular activity)? | many laboratories. We also agree that quantification is challenging for broth microdilution. Still, we showed a very high agreement in the visual reading during the AMST multicentre study, especially when growth in the 1:100 control was used as a cut off for reading the visual MIC. For the implementation of other reading methods such as those described in Rancoita PM. et al AAC 2017 a calibration will be required.  

**Action:** No change required. |
| --- | --- |
| 2. The experience from the EUCAST reference methods for fungi and rapidly growing bacteria is that there may indeed be methodological exceptions similar to those described for some compounds. If such issues are encountered, new versions of the reference method describing those potential exceptions will be developed.  

**Action:** No change required. |
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<td>Otsuka - <a href="mailto:kelly.stinson-CW@otsuka-us.com">kelly.stinson-CW@otsuka-us.com</a> 25th of June 2019</td>
<td>Reference protocol, step 1.7 Recommend defining “highest concentration” by adding the concentration in parenthesis as it relates to Appendix 1. In other words, it is assumed that C1 is the highest concentration when looking at the appendix, so we suggest modifying to: “Add 0.1 mL of the 4X working solution to the wells corresponding to the highest concentration (C1)....” Step 1.8 Suggest changing “suspension” to “antibiotic solution”.</td>
<td>Agreed. Action: The protocol was revised according to the suggestion.</td>
</tr>
<tr>
<td>Otsuka - <a href="mailto:kelly.stinson-CW@otsuka-us.com">kelly.stinson-CW@otsuka-us.com</a></td>
<td>Reference protocol, step 1 We suggest adding details on stability of plates once agent is added to the media. Must they be used on the same day, or can they be stored? If so, for what period of time and under what conditions?</td>
<td>The plates need to be used the same day and should be prepared freshly as for other EUCAST reference methods. This was specified in step 1. If other conditions like freezing the plates are to be applied, this needs to be verified/calibrated against the reference method.</td>
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<tr>
<td>Action</td>
<td>The protocol was clarified as described above.</td>
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<td>-----------------------------------------------</td>
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<tr>
<td>Otsuka - <a href="mailto:kelly.stinson-CW@otsuka-us.com">kelly.stinson-CW@otsuka-us.com</a></td>
<td>Reference protocol, step 2.2</td>
<td></td>
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<td></td>
<td>• Suggest adding more detail regarding the recommendation not to use the reference strain beyond three passages. US ATCC allows up to 5 passages.</td>
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<td></td>
<td>• Suggest adding details on preparing inoculum direct from liquid culture as well.</td>
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<td></td>
<td>1. <strong>Action:</strong> Step 2.2 was revised to allow 5 passages according to the ATCC guidelines.</td>
<td></td>
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<tr>
<td></td>
<td>2. The reference method will only include the possibility to use solid media cultures to minimize methodological variability. <strong>Action:</strong> No change required.</td>
<td></td>
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<tr>
<td></td>
<td>3. Step 2.3 has been tested and widely discussed within EUCAST AMST and the consensus is that the suggested method gives the most stable inoculum. Briefly, colonies need to be dissociated with the glass beads and this is more adequately done when the colonies stay dried, i.e. without any liquid. <strong>Action:</strong> No change required.</td>
<td></td>
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<td></td>
<td>4. In step 2.4, dH2O was regarded as more stable for preparing the inoculum (a critical step for stability of the method) than 7H9 media. Tween saline is not used as it is regarded to disturb the interaction with some antibiotics. <strong>Action:</strong> No change required.</td>
<td></td>
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**EUCAST form for General Consultation**

<table>
<thead>
<tr>
<th>Step 2.8</th>
<th>5. Agreed.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suggest clarifying that inoculum should be added to well with lowest drug concentration first (column D), and then proceeding upward to the highest concentration.</td>
<td><strong>Action:</strong> It was clarified that the inoculum should be added to the well with lowest inoculum first in step 2.9.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Otsuka - <a href="mailto:kelly.stinson-CW@otsuka-us.com">kelly.stinson-CW@otsuka-us.com</a></th>
<th>More guidance needed on the use of CFU control plate - read at 21 days vs 14 days for broth plates. Would results not be issued until Day 21 control plate is valid?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Can broth plates be held for 21 days if GC 1% has no growth at Day 14?</td>
</tr>
<tr>
<td></td>
<td>1. As stated in 2.8, plates for CFU control may be read from day 14. The test is not valid if the inoculum is outside the specified range.</td>
</tr>
<tr>
<td><strong>Action:</strong> No change required.</td>
<td>2. Agreed.</td>
</tr>
<tr>
<td><strong>Action:</strong> It has been clarified in 3.2 that reading at 21 days is a possibility if there is no growth at GC1% at day 14.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Otsuka - <a href="mailto:kelly.stinson-CW@otsuka-us.com">kelly.stinson-CW@otsuka-us.com</a></th>
<th>Will EUCAST provide sample worksheets for reporting of results and capturing of QC?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>There is no current plan in providing worksheets for reporting results and capturing of QC which EUCAST AMST believe could be handled in most routine laboratories according to their current procedures.</td>
</tr>
<tr>
<td>Comment from (name, contact details)</td>
<td>Comments</td>
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<tr>
<td>-------------------------------------</td>
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</tr>
<tr>
<td>Richard Pfeltz, <strong>BD Life Sciences</strong> Sparks, MD, USA <a href="mailto:Richard.Pfeltz@bd.com">Richard.Pfeltz@bd.com</a> 26th of June 2019</td>
<td>typo in items 2.2, 2.4, 2.8, table 1 &amp; its footnotes, and appendix 1: change $dH_20$ to $dH_2O$ (replace zero with letter “O”)</td>
</tr>
</tbody>
</table>
| Item 2.4: Is densitometer the correct term to use?  
1. Densitometry is the measurement of liquid density  
2. Optical density is a measure of turbidity and is done with a photometer or nephelometer…perhaps re-phrase the sentence to clarify.  
3. How many different turbidity-measurement devices have been tested for inoculum preparation? | 1-2. We recommend to use a densitometer in this context and the term is used by several companies. Such devices, also called suspension turbidity meters, are also used for other EUCAST reference methods.  
**Action:** It was clarified that densitometers are used to measure suspension turbidity in 2.5.  
3. There were different turbidity-measurement devices available in the four participating AMST laboratories. As long as they are used according to the manufacturer and produce an inoculum according to the CFU control (as they did in |
<table>
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<th>EUCAST form for General Consultation</th>
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<tbody>
<tr>
<td>4. The McFarland standards are generally used clinically vs. OD at a given wavelength for spectrophotometers that are generally used in academic research labs. A McFarland-to-OD&lt;sub&gt;xxx nm&lt;/sub&gt; conversion factor is needed if both are allowed.</td>
</tr>
<tr>
<td>the internal EUCAST AMST study), they may be used.</td>
</tr>
<tr>
<td><strong>Action:</strong> No change required.</td>
</tr>
<tr>
<td>4. Even if McF standards have been used traditionally to visually prepare inoculums for the MTBC, spectrophotometers/densitometers are used for all other bacteria and fungi in both academic and routine laboratories as it resulted in a reproducible inoculum. Thus, a visual McF-standard should not be used according to the reference protocol.</td>
</tr>
<tr>
<td><strong>Action:</strong> No change required.</td>
</tr>
<tr>
<td>Item 2.7:</td>
</tr>
<tr>
<td>Last sentence is unclear. From plate count expectations, back-calculation of the titer of the 0.5 McFarland suspension is expected to be 5E6-5E7 CFU/mL. So the protocol is trying to say that the 1E-2 dilution from the 0.5 McFarland suspension is 1E5 CFU/mL (range 5E4-5E5 cfu/mL)?</td>
</tr>
<tr>
<td>1. Agreed.</td>
</tr>
<tr>
<td><strong>Action:</strong> The last sentence was clarified accordingly.</td>
</tr>
<tr>
<td>Items 2.5-2.9: This is very “text-intense”. Please consider adding an Appendix 2 that would include a figure of a dilution scheme to help users follow this better/more accurately (see example below).</td>
</tr>
<tr>
<td>2. It is a valid suggestion to add a figure to visualise steps 2.5-2.9. This will be considered for future revisions of the reference method and a planned publication.</td>
</tr>
</tbody>
</table>
### Item 3.2: is the test invalid if one or more of the plate counts come out above or below their expected values? What if only the 1E-3 inoculum check count is outside of the expected range?

**Action:** No change required in this version of the protocol but will be considered for future revisions.

The test is valid if the inoculum check is within $5 \times 10^4$-$5 \times 10^5$ CFU/mL. The dilutions 1E-2, 1E-3 and 1E-4 are included to ensure a plate with countable colonies. It is probably very unlikely that the 1:10 dilutions will not be correctly performed and if so, the test should be repeated if countable colonies are not present on the other dilutions.

**Action:** No change required.

---

### No guidance on or prohibition against the use of redox indicators?

**Action:** No change required.

The current version of the reference method is developed for use without redox indicators, since those indicators are measuring metabolic activity and not growth. The use of redox indicators would require calibrating the technique by following the EUCAST calibration SOP.

**Action:** No change required.

---

### No guidance on interpretation of trailing endpoints?

1. The guidance for non-mycobacterial bacteria is generally call the MIC at the drug concentration yielding growth ≤ 80% that of the drug-free control well, which is highly subjective with

   We agree that trailing endpoint may be a problem for mycobacterial susceptibility testing and in particular for NTM. So far, there has not been any need of guidance of interpretation of trailing endpoints.
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<table>
<thead>
<tr>
<th>homogeneous suspensions and questionable whether it can be applied to Mtbc growth</th>
<th>which is similar to what has been experienced by others for the MTBC (Rancoita PM. et al AAC 2017). The NTMs will be addressed separately.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Very weak drugs such as PAS and thioamides will not give distinct endpoints in terms of growth/no growth</td>
<td>Action: No change required.</td>
</tr>
<tr>
<td>2. The inclusion of a QC isolate (H37Rv ATCC 27294) which should always be in the range will facilitate the interpretation for some drugs, as mentioned. If complementary guidance would be needed, this will be addressed in future revisions of the reference method.</td>
<td>Action: No change required.</td>
</tr>
<tr>
<td>Small volumes for Mtbc dilutions are a notorious source of variability, and the protocol’s 100-fold dilutions in two 10-fold steps is no doubt because of this. However, 10-µL inoculating loops are specified for platings that seem to just be looking for growth and doesn’t specify colony count values. There is concern about in-spec inocula with no growth from the GC1% on plates but growth in microwells.</td>
<td>3. We agree that 100-fold dilutions should be avoided if the aim is to produce a stable inoculum. In the validation study, there has been no issues with “no growth” from the GC1% wells up to 14 days with the currently recommended inoculum.</td>
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<tr>
<td><em>Action:</em> No change required.</td>
<td><em>Action:</em> No change required.</td>
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### EUCAST form for General Consultation

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<tr>
<th>Comment from (name, contact details)</th>
<th>Comments</th>
<th>EUCAST Responses</th>
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<tbody>
<tr>
<td>WHO (Chris Gilpin and Max Salfinger) 26th of June 2019 and 1st of July 2019</td>
<td>1. The endorsement of a methodology with biosafety concerns is problematic (using a liquid medium in a 96 well plate format requires additional precautions) and EUCAST should provide some additional guidance for laboratories working with a methodology with inherently more risk. This is currently missing from the documents I see on the website.</td>
<td>1. The discussion on biosafety is indeed highly relevant. The biosafety issue in any TB laboratory performing DST for MTBC should be at the level suitable for working with liquid or solid media cultures. This is clearly essential, but detailed biohazard recommendations for TB culture are regarded to be beyond scope of the actual reference method document. For the reference method, the microplates should be prepared and covered in safety cabinets and then incubated as detailed. The reading should also be done under safety cabinets. Additionally, the actual quantity of MTBC cultured in the laboratory may be less using broth microdilution than with broth macrodilution.</td>
</tr>
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</table>
Many 7H9 methodological variants exist (incl. Janssen plates, Cryptic plates, CLSI method), and there will be a great need for the standardization of these other 7H9 methods against the EUCAST methodology. To this effect, it is worth noting that many of these plates have important limitations that will make standardization with this protocol difficult. For example, the Cryptic plates would need to be calibrated against new QC strains given that there are major MIC truncations for many of the drugs included in the plates.

There are also no currently established breakpoints for most drugs on 7H9. In short, 7H9 is useful as a

Action: The importance of biosafety precautions in general were added to the protocol.

We agree that there is a need to carefully standardize any other 7H9 format for MIC testing against the EUCAST MIC reference method (7H9 broth microdilution). A calibration SOP will be provided along with the final version of the reference method. Indeed, truncation must be avoided to ensure proper quality control and this is also important for the calibration/validation. The issue of truncation and meticulous quality control should also include MIC testing in routinely used DST devices such as 7H10, LJ and MGIT.

Action: No change required.

Clearly, there is a need to establish evidence based breakpoints in the reference method, which is one reason
<table>
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<th></th>
<th>calibration and research tool, but its clinical utility currently is questionable</th>
<th>why the method has been developed. From the EUCAST standpoint, there cannot be different clinical breakpoints with regard to the medium or the method. From now on, EUCAST will establish clinical breakpoints for the MTBC according to wild-type MIC distributions along with clinical outcome data and PK/PD-data. Any other currently used method for routine susceptibility testing of MTBC (such as MGIT) will need to be carefully calibrated against the reference method according to the EUCAST calibration SOP. Then this method/technique may be used along with EUCAST breakpoints.</th>
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<tbody>
<tr>
<td>4.</td>
<td>This should be considered a stand-alone document with all the bells and whistles, e.g.,</td>
<td><strong>Action:</strong> No change required.</td>
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<tr>
<td></td>
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<td>4. The reference method should be possible to perform with most media suppliers and consumables as long as the specifications</td>
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<tr>
<td>section on biosafety is missing; list of consumables and suppliers and contact information/website (if specified, such as Sigma, ATCC, etc.); QC of OADC is missing.</td>
<td>in the method are adhered to. When QC-ranges and ECOFFs are defined in the reference method, several media distributors including those of OADC needs to be included. However, those studies are beyond scope of the actual reference protocol. The biosafety issue in any TB laboratory should be at the level suitable for working with liquid or solid media cultures of Mtb.</td>
<td></td>
</tr>
<tr>
<td>5. Ad 1.3: What is the rationale to add OADC at 50°C? It could be added also at room temperature.</td>
<td>Action: No change required.</td>
<td></td>
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<tr>
<td>5. We have chosen to follow the instructions by most manufacturers. In our hands, adding OADC at 50°C works fine. If OADC is to be used at RT, which may be easier, this needs to be validated against the current version of the reference method.</td>
<td>Action: No change required.</td>
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<td>6.</td>
<td>Ad 2.2: The 3 sentences starting with ‘Bacterial colonies … scrapping off media.’ need to be moved to the first part of 2.3</td>
<td>6. Agreed. <strong>Action:</strong> We have moved the 3 sentences to a separate point which was inserted (2.3).</td>
</tr>
<tr>
<td>7.</td>
<td>Ad 2.4: Start off the section stating that “The supernatant, without disturbing the sediment, is transferred into a new sterile tube. Adjust the optical density of the suspension...”</td>
<td>7. Agreed. <strong>Action:</strong> The protocol was revised to specify that the McF of the supernatant should be determined.</td>
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<tr>
<td><strong>Minor comments:</strong></td>
<td><strong>Action:</strong> Corrected</td>
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<tr>
<td>Use the term ‘7H9 broth’ instead of ‘7H9 media’ throughout the document, where appropriate. Media would also include agar… if term is not changed, then it should read ‘7H9 medium’ singular and not plural (e.g., “media” in title)</td>
<td><strong>Action:</strong> Corrected</td>
<td></td>
</tr>
<tr>
<td>Ad 2.1: Instead of “RT” use 18-22°C</td>
<td><strong>Action:</strong> Added according to suggestion.</td>
<td></td>
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<tr>
<td>Ad 2.2: Add Middlebrook agar to “7H10 or 7H11 Middlebrook agar, LJ or other egg-based solid media”)</td>
<td><strong>Action:</strong> Added according to suggestion.</td>
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<tr>
<td>Ad 2.2: Add plastic loop – ‘an applicator or plastic loop’</td>
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<tr>
<td>Ad 2.3: CLSI states 1-2 minutes for vortexing; if exact 2 minutes of vortexing is indeed important, then state so. [FYI, Kent &amp; Kubica CDC 1985 states even 5 to 10 minutes. The step of homogenizing the inoculum is very critical. It begs the question, if one should state as a compromise 3 to 5 minutes instead of 2 minutes or 1-2 minutes?]</td>
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<tr>
<td><strong>Action:</strong> Revised partly according to the suggestion. The “2 minutes” is kept since we agree that the homogenizing step is very important.</td>
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<tr>
<td>Ad 2.3: Instead of ‘careful closing of the cap’ suggest ‘Close the cap tightly and homogenize the tube’s content by vigorously vortexing the tube for 1-2 minutes’ [CLSI].</td>
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<tr>
<td><strong>Action:</strong> Revised according to suggestion</td>
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<tr>
<td>Instead of ‘When colonies are well disaggregated, add....’ state ‘When clumps are well dispersed, add...’</td>
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<td><strong>Action:</strong> Revised according to suggestion</td>
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<tr>
<td>Ad 2.4: McFarland and densitometer are measuring the same, although the densitometer is more accurate; however, not every laboratory has one. How important is it to use a densitometer?</td>
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</tr>
<tr>
<td>We believe that a densitometer should be used and that we should be as strict for the MTBC as is the case when the inoculum is prepared for other bacteria and fungi. <strong>Action:</strong> No change required.</td>
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<tr>
<td>Ad 2.6: Spell out GC first time used.</td>
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<td><strong>Action:</strong> Corrected.</td>
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<tr>
<td>Ad 2.7: The step described in this section is performed separately, and the results are not obtained before the step 2.8 is performed – should be moved elsewhere.</td>
<td></td>
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<tr>
<td>We prefer this order of procedure to stress the importance of doing the inoculum control at the same time as the plates are inoculated. <strong>Action:</strong> No change required.</td>
<td></td>
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<tr>
<td>AD 2.7: CFU count-instead of plating just one 10 µL, recommend 3 spots with each 10 µL and then calculate the average for each dilution.</td>
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<tr>
<td>During the development of the method, we found that one spot was enough to establish that the inoculum was within range but as this part is important to assess we will investigate whether three spots would increase the accuracy of the estimate and consider this strategy for future revisions.</td>
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<tr>
<td>Action: No change required.</td>
<td></td>
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<tr>
<td>Ad 3.1: Characteristic of plastic bags O₂- or CO₂-permeable, or? Please define.</td>
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</tr>
<tr>
<td>Action: It was specified that permeable plastic bags may be used but boxes may be even easier from practical and safety aspects.</td>
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<tr>
<td>Ad 3.2: Recommend the readings at 7, 10, and 14 days, not just 7 and 14 days.</td>
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<tr>
<td>We prefer to stick to the recommended reading at 7 and 14 days, as this was included in the validation study. If enough growth was not visible at day 14 in the GC1% we have included a possibility to read the plates at a maximum of 21 days.</td>
<td></td>
<td></td>
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<tr>
<td>Action: A possibility to read the plates up to 21 days was included.</td>
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</table>
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