

Media preparation for EUCAST disk diffusion testing and for determination of MIC values by the broth microdilution method

Changes from previous version (v. 5.0)

A. Media for disk diffusion testing

Section	Change
Introduction	Requirements for Mueller-Hinton media added.

B. Media for MIC determination by the broth microdilution method

Section	Change
Introduction	ISO standard 20776-1 updated to 2019 version.
Introduction	Requirements for Mueller-Hinton broth added.

A. Media for disk diffusion testing

Mueller-Hinton (MH) agar and MH agar supplemented with defibrinated horse blood and β -NAD (MH-F)

MH agar, un-supplemented Mueller-Hinton agar, is used for testing of non-fastidious organisms.

MH-F agar, MH supplemented with 5% mechanically defibrinated horse blood and 20 mg/L β -NAD, is used for testing *Streptococcus* spp. (including *S. pneumoniae*), *Haemophilus influenzae*, *Moraxella catarrhalis*, *Listeria monocytogenes*, *Campylobacter jejuni* and *coli*, *Pasteurella multocida*, *Corynebacterium* spp., *Aerococcus sanguinicola* and *urinae* and *Kingella kingae*.

Agar plates may be prepared in-house from dehydrated media or purchased ready-poured from commercial sources. Dehydrated Mueller-Hinton media should meet the requirements in the ISO Technical specification, ISO/TS 16782, 2016 and the quality control criteria published by EUCAST.

MH and MH-F agar plates are prepared as follows:

1. Reagents	
1.1	MH agar powder from commercial source.
1.2	Mechanically defibrinated horse blood.
1.3	β -Nicotinamide adenine dinucleotide (β -NAD), purity $\geq 98\%$.

2. Preparation of β -NAD stock solution	
2.1	Dissolve β -NAD in sterile deionized water to a concentration of 20 mg/mL.
2.2	Sterilize the solution through a 0.2 μ m membrane filter.
2.3	The stock solution may be stored at -20°C in aliquots and defrosted as required. Do not refreeze unused solution.

3. Preparation of agar plates	
3.1	Prepare and autoclave MH agar according to the manufacturer's instructions.
3.2	Cool medium to 42-45°C.
3.3	For MH-F, aseptically add 50 mL mechanically defibrinated horse blood and 1 mL β -NAD stock solution per litre medium. Mix well and dispense immediately.
3.4	Dispense medium into sterile Petri dishes to give a level depth of 4 ± 0.5 mm (approximately 25 mL in a 90 mm circular plate, 31 mL in a 100 mm circular plate, 71 mL in a 150 mm circular plate, 40 mL in a 100 mm square plate). Ascertain that a correct volume, based on the true dimensions of the Petri dish in use, is calculated. Plate dimensions may differ between manufacturers.
3.5	Allow the agar to set before moving the plates.
3.6	The surface of the agar should be dry before use. No drops of water should be visible on the surface of the agar or inside the lid. If necessary, dry plates either at 20-25°C overnight, or at 35°C, with the lid removed, for 15 min. Do not over-dry plates.

4. Storage of agar plates	
4.1	Store plates prepared in-house at 4-8°C.
4.2	For plates prepared in-house, plate drying, storage conditions and shelf life should be determined as part of the laboratory quality assurance programme.
4.3	Commercially prepared plates should be stored as recommended by the manufacturer and used within the labelled expiry date.
4.4	For agar plates (commercially or in-house prepared) stored in plastic bags or sealed containers, it may be necessary to dry the plates prior to use. This is to avoid excess moisture, which may result in problems with fuzzy zone edges and/or haze within zones.

5. Quality control	
5.1	Use a surface pH electrode to check that the pH is within the range 7.2-7.4.
5.2	Check that the agar depth is 4 ± 0.5 mm.
5.3	Check that the medium supports good growth of control strain(s) of the intended test organisms.
5.4	Perform disk diffusion for quality control strains according to EUCAST recommendations and check that inhibition zones are within acceptable ranges for all bacteria-antimicrobial agent combinations used (EUCAST QC tables).

B. Media for MIC determination by the broth microdilution method

Cation-adjusted Mueller-Hinton broth (MHB) and MHB supplemented with lysed horse blood and β -NAD (MH-F broth)

MH broth, un-supplemented cation-adjusted Mueller-Hinton broth, is used for testing of non-fastidious organisms according to the ISO standard 20776-1, 2019.

MH-F broth, cation-adjusted MH broth supplemented with 5% lysed horse blood and 20 mg/L β -NAD, is used for testing *Streptococcus* spp. (including *S. pneumoniae*), *Haemophilus influenzae*, *Moraxella catarrhalis*, *Listeria monocytogenes*, *Campylobacter jejuni* and *coli*, *Pasteurella multocida*, *Corynebacterium* spp., *Aerococcus sanguinicola* and *urinae*, *Kingella kingae* and several other fastidious organisms.

Un-supplemented MH broth may be purchased from commercial sources or prepared locally according to the manufacturers' instructions. MH broth should meet the requirements in the ISO Technical specification, ISO/TS 16782, 2016 and the quality control criteria published by EUCAST.

MH-F broth is prepared as follows:

1. Reagents	
1.1	Cation-adjusted MHB from commercial source.
1.2	50% lysed horse blood.
1.3	β -Nicotinamide adenine dinucleotide (β -NAD), purity \geq 98%.

2. Preparation of 50% lysed horse blood stock solution	
2.1	Aseptically dilute mechanically defibrinated horse blood with an equal amount of sterile deionized water.
2.2	Freeze the blood at -20°C overnight and thaw. Repeat the cycle until the cells are completely lysed (three cycles is usually sufficient but the ISO standard 20776-1 suggests that up to seven cycles may be required).
2.3	Clarify the 50% lysed horse blood by centrifugation and discard the pellet. A clear solution is essential for reading. Failure to clarify the solution may be due to inadequate lysis or centrifugation. Repeating the centrifugation may improve the clarity of the solution.
2.4	The stock solution may be stored at -20°C in aliquots and defrosted as required. Do not refreeze unused solution.

3. Preparation of β-NAD stock solution	
3.1	Dissolve β -NAD in sterile deionized water to a concentration of 20 mg/mL.
3.2	Sterilize the solution through a 0.2 μ m membrane filter.
3.3	The stock solution may be stored at -20°C in aliquots and defrosted as required. Do not refreeze unused solution.

4. Preparation MH-F broth	
4.1	Prepare and autoclave cation-adjusted MHB according to the manufacturer's instructions, but with 100 mL less deionized water per litre to allow for the addition of lysed horse blood.
4.2	Cool medium to 42-45°C.
4.3	Aseptically add 100 mL 50% lysed horse blood and 1 mL β -NAD stock solution per litre medium and mix well.
4.4	Dispense MH-F broth in sterile containers with screw caps.

5. Storage of MH-F broth	
5.1	Store MH-F broth at 4-8°C.
5.2	Storage conditions and shelf life should be determined as part of the laboratory quality assurance programme. A shelf life of 3 months can be expected.

6. Quality control	
6.1	Check that the pH is within the range 7.2-7.4.
6.2	Check that the medium supports good growth of control strain(s) of the intended test organisms.
6.3	Check that MICs are within control limits for all bacteria-antimicrobial agent combinations used (EUCAST QC tables).