EUCAST disk diffusion methodology for selected rapidly growing anaerobic bacteria* on Fastidious Anaerobe Agar with defibrinated horse blood (FAA-HB)

* This method is validated for 16-20 h incubation of ***Bacteroides* spp.**, ***Prevotella* spp.**, ***Fusobacterium necrophorum***, ***Clostridium perfringens*** and ***Cutibacterium acnes***. It cannot be used for other species of anaerobic bacteria or incubation beyond 20 h.

Changes from previous version (1.0)

Section	Change
General	The abbreviation for the media changed from FAA to FAA-HB
Medium, 1c	The expected shelf life for in-house produced FAA-HB changed from 14 to 21 days
Inoculation, 1a **Troubleshooting, 1b**	New recommendation to remove excess fluid from the cotton swab before streaking plates for *Clostridium perfringens*
Inoculation, 2a **Troubleshooting, 1b**	Information added that it is particularly important to streak plates carefully also for some *Prevotella* spp. growing with small colonies on FAA-HB
Troubleshooting, 1a	Information on composition of commercial FAA-HB plates added
Troubleshooting, 1c	Additional information on number of disks added per plate added
QC tables	Quality control criteria for anaerobic QC strains moved from this document to the EUCAST QC Tables v. 13.0

Medium

1. Use Fastidious Anaerobe Agar with 5% mechanically defibrinated horse blood (FAA-HB) and no other additives.
    a. The agar depth must be 4.0 ± 0.5 mm.
    b. For in-house preparation of plates, allow the medium to cool to 42-45°C before adding the blood.
    c. In-house produced plates should be stored at 4-8°C in ventilated racks and protected from light. The shelf life should be determined as part of the laboratory quality assurance programme, but a minimum shelf life of 21 days can be expected.
    d. Commercially prepared plates should be stored as recommended by the manufacturer, protected from light and used before the expiry date.
    e. The FAA-HB plates must be dried prior to inoculation to avoid excess moisture, which may result in fuzzy zone edges, swarming and/or haze within zones. One of the following procedures can be used:
        i. Dry at 20-25°C overnight or
        ii. Dry at 35°C, with the lid removed, for 15 min. The plates must reach room temperature before this step.
        iii. For plates stored in plastic bags or sealed containers, it might be necessary to dry plates first according to (i) followed by (ii).
    f. Do not pre-reduce FAA-HB plates in an anaerobic environment before use.

Inoculum preparation

1. Use a sterile loop or a cotton swab to pick colonies from an anaerobic overnight culture on non-selective media. Use several morphologically similar colonies.
2. Suspend the colonies in 0.85% saline and mix to an even turbidity.
3. Adjust the density of the inoculum suspension to McFarland 1.0 (0.9-1.1) by adding saline or more bacteria. The use of a photometric device is recommended.
4. Use the inoculum suspension **within 15 minutes** of preparation.

Inoculation of agar plates

1. Dip a sterile cotton swab into the McFarland 1.0 suspension.
    a. For *Bacteroides* spp. and *Clostridium perfringens*, remove excess fluid by turning the swab against the inside of the tube to avoid over-inoculation.
2. Spread the inoculum evenly over the entire agar surface, ensuring that there are no gaps between streaks.
    a. This is particularly important for species growing with small colonies on FAA-HB, such as *Cutibacterium acnes* and some *Prevotella* spp.

A correctly inoculated agar plate will result in a confluent lawn of growth with uniformly circular inhibition zones.

Application of antimicrobial disks

1. Use EUCAST recommended disk potencies as listed in the EUCAST Breakpoint Tables or Quality Control Tables.
2. Allow disks to reach room temperature before opening cartridges or containers.
3. Apply disks **within 15 minutes** of inoculation.
    a. Disks must be in close and firm contact with the agar surface and must not be moved once they have been applied.
    b. To avoid overlapping of zones, the number of disks on a plate must be limited. Optimally, use no more than three disks on a 90-mm circular plate (four disks can be used for *Bacteroides* spp.).

Incubation of plates

1. Invert plates and make sure disks do not fall off the agar surface. Incubate **within 15 minutes** of disk application.
2. Incubate FAA-HB plates in an anaerobic environment at 35-37°C for 16-20 h.
    a. Anaerobic conditions can be achieved either in an anaerobic work station, or in jars with anaerobic gas-generating envelopes or a gas-generating system such as the Anoxomat.
    b. Prolonged incubation (beyond 20 h) is not allowed as it will affect zone sizes and invalidate interpretative criteria.

Reading of inhibition zones

1. A correct inoculum should result in a confluent lawn of growth evenly distributed over the agar surface. If growth is non-confluent, the test must be repeated, or an MIC method may be used.
2. Read plates from the front with the lid removed and with reflected light.
3. Read zone edges at the point of complete inhibition as judged by the naked eye with the plate held approximately 30 cm from the eye at a 45-degree angle to the work bench.
4. Measure the inhibition zone diameters to the nearest millimetre with a ruler or a calliper.
    a. If haze within the zone occurs, read the most obvious zone edge. Tilt the plate towards you to better define the obvious zone edge.
    b. In case of double zones, read the inner zone edge.
    c. Ignore haemolysis and swarming when reading zones.
5. Isolated colonies within the inhibition zone should be taken into account. **For clindamycin, it is particularly important to examine zones carefully for colonies growing within the zone.**
6. Pictures with reading examples are available in the Reading Guide for disk diffusion of anaerobic bacteria on FAA-HB.

Quality control

1. Perform quality control (QC) at each test occasion. Use an overnight culture of the QC strain and follow the same testing procedure as for clinical isolates.
    a. Use *Bacteroides fragilis* ATCC 25285 and *Clostridium perfringens* ATCC 13124 to monitor the test performance. For QC ranges and targets, see EUCAST QC Tables.
    b. Use *Clostridium perfringens* DSM 25589 with a metronidazole 5 µg disk to monitor the anaerobic atmosphere. This combination has been shown to be a sensitive indicator of the anaerobic atmosphere. Insufficient anaerobicity may affect growth and susceptibility test results for anaerobic bacteria. For interpretive criteria, see EUCAST QC Tables.
        i. The anaerobic atmosphere of workstations requires special attention. Regular service and technical control are necessary.

Troubleshooting

1. There may be one or several reasons for QC results out of range. Strict adherence to the protocol is required to ensure reliable results. Troubleshooting guidance:
    a. Media
        i. Are FAA-HB plates stored and dried according to the instructions above?
        ii. Is the agar depth 4.0 ± 0.5 mm? The target agar depth is 4.0 mm and ± 0.5 mm is allowed to account for random, but not systematic deviations.
        iii. Are commercial FAA-HB plates prepared according to EUCAST recommendations with 5% mechanically defibrinated horse blood as the only supplement?
    b. Streaking of plates
        i. Make sure that the inoculum is spread evenly over the entire agar surface, ensuring that there are no gaps between streaks.
            1. This is particularly important for species growing with small colonies on FAA-HB such as *Cutibacterium acnes* and some *Prevotella* spp..
        ii. For *Bacteroides* spp. and *Clostridium perfringens*, make sure to remove excess fluid by turning the swab against the inside of the tube to avoid over-inoculation.
    c. Antimicrobial disks
        i. Limit the number of disks on the agar surface to allow uninhibited growth and avoid overlapping of zones. For most species and antimicrobial agents, three disks can be used on a 90-mm circular plate, but for some isolates it might be as low as two disks per plate.

   ii. Allow disks to reach room temperature before opening cartridges and make sure to adhere to recommendations for storage of disks.

d. Incubation
   i. Check the anaerobic atmosphere (irrespective of how it is created) regularly.
      1. The anaerobic atmosphere of workstations requires regular service and technical control. The atmosphere and temperature can be affected by how often the system is opened for loading and unloading of plates as well as the amount of plates in the workstation.
      2. When using jars for anaerobic incubation, make sure that there is no leakage.
   ii. EUCAST breakpoints and QC criteria for disk diffusion of anaerobes on FAA-HB are validated for 16-20 h incubation only.
      1. Prolonged incubation is not allowed as it will affect zone sizes significantly.
e. Reading of zones
   i. Make sure to follow the specific reading instructions for anaerobes as listed above. Pictures with examples are available in the Reading Guide for disk diffusion of anaerobic bacteria on FAA-HB.