INTENDED USE
Etest is a quantitative technique for determining the antimicrobial susceptibility of Gram-negative and Gram-positive aerobic bacteria such as Enterobacteriaceae, Pseudomonas, Staphylococcus and Enterococcus species and fastidious bacteria, such as anaerobes, N. gonorrhoeae, S. pneumoniae, Streptococcus and Haemophilus species. The system comprises a predefined antibiotic gradient which is used to determine the Minimum Inhibitory Concentration (MIC), in µg/mL, of different antimicrobial agents against microorganisms as tested on agar media using overnight incubation.

SUMMARY AND EXPLANATION
Current Antimicrobial Susceptibility Testing (AST) methods are based either on quantitative dilution techniques or qualitative diffusion procedures. Dilution methods are based on two-fold serial dilutions of antibiotics in broth or agar media. These methods generate the MIC value i.e. Minimum Inhibitory Concentration of a given antibiotic in µg/mL that will inhibit the growth of a particular bacterium under defined experimental conditions.

PRINCIPLES OF USE
The Etest gradient technology is based on a combination of the concepts of dilution and diffusion principles for susceptibility testing. As with other dilution methods, Etest directly quantifies antimicrobial susceptibility in terms of discrete MIC values. However, in using a predefined, stable and continuous antibiotic concentration gradient, Etest MIC values can be more precise and reproducible than results obtained from conventional procedures based on discontinuous two-fold serial dilutions.

Etest is a thin, inert and non-porous plastic strip. One side of the strip (A) carries the MIC reading scale in µg/mL and a two or three-letter code on the handle to designate the identity of the antibiotic. A predefined exponential gradient of antibiotic, dried and stabilised, is immobilised on the other side of the strip (B) with the concentration maximum at a, and the minimum at b (Figure 1). The gradient covers a continuous concentration range across 15 two-fold dilutions of a conventional MIC method.

To obtain reproducible MICs from a gradient based system, the stability of the gradient must be maintained throughout the critical period when the position of the growth/inhibition edge for a particular bacterium/antibiotic combination is determined. Due to the stability and precision of the Etest predefined gradient, MIC values have been shown to be reproducible and equivalent to those of the CLSI® reference dilution procedures.

REAGENTS
Etest is supplied in a package of 30 or 100 test strips (depending on package format) of one antimicrobial agent.

STORAGE
Etest should always be stored according to the temperature specified on the packaging, until the given expiry date. Products can always be stored lower than the maximum temperature specified.

Etest gradient strips left over from an opened package must be kept dry. The opened package should be either re-sealed with a sealing clamp or placed in an airtight storage container with active desiccant, and stored within the temperature range stated on the label. Left-over strips in storage containers can be used until the expiry date if correctly stored and handled. Ensure that the batch number and expiry date are marked on the storage container. Protect Etest strips from moisture, heat and direct exposure to strong light at all times.

Prevent moisture from penetrating into or forming within the package or storage container. Etest strips must be kept dry with active desiccant.

HANDLING
• Before using the Etest gradient strips from an unopened package, visually inspect to ensure the package is intact. Do not use the Etest gradient strips if the package has been damaged.

• When removed from the refrigerator/freezer, allow the original package or storage container to reach room temperature before opening (+4 °C/ approx. 15 minutes, -20 °C/ approx. 30 minutes). Ensure that moisture condensing on the outer surface has evaporated completely before opening the package. Packages stored at room temperature can be used immediately.

• Opening instructions:
  • Single Pack (refer to diagram below)
    1. Hold the packaging between the thumb and the index finger, placing the thumb tip on the indented area on the back.
    2. Press forward with the thumb and back with the index finger to break open the aluminium film, ensuring that the desiccant remains in the top part of the packaging.
    3. Bend the top part back to open the packaging completely.
    4. Remove the Etest strip from the packaging using forceps or other manual applicator.

  • Blister
    - Open one blister compartment by cutting the packaging along the dotted line using scissors.
  • Foam
    - Open the packaging by cutting off one end of the aluminium pouch using scissors.

Figure 1: Etest gradient configuration
When an Etest gradient strip is applied to an inoculated agar surface, there is immediate and effective transfer of the preformed antibiotic gradient on the plastic carrier surface into the agar matrix. A stable, continuous and exponential gradient of antibiotic concentrations is formed directly underneath the strip. After incubation, whereby bacterial growth becomes visible, a symmetrical inhibition ellipse centred along the strip is seen. The MIC value is read from the scale in terms of µg/mL where the pointed end of the ellipse intersects the strip.
PROCEDURES

Materials provided

- 30 or 100 Etest strips of one antibiotic
- 1 package insert + TABLE 1 provided in the kit or downloadable from www.biomerieux.com/techlib

Materials required but not provided

- Agar plates (90 mm or 150 mm) with the appropriate susceptibility test media
- Inoculum suspension media
- Swabs (sterile, non-toxic and not too tightly spun), test tubes, and scissors
- Manual applicator [e.g., Mini Grip-It (bioMérieux Ref. 411200), forceps or similar device] or bioTools [Retco C80™ (bioMérieux Ref. 559803), Nema C88™ (bioMérieux Ref. 559804), Simplex C76™ (bioMérieux Ref. 559802)]
- 0.5 and 1 McFarland turbidity standards (bioMérieux Ref. 70 900) or DENSIMAT (bioMérieux Ref. 99 234)
- Incubator (35 ± 2 °C), anaerobic jar or chamber or CO₂ incubator
- Manual applicator, Nema C88 (Figure 5) or Simplex C76 (Figure 6). Position the Etest gradient strip with the MIC scale facing upwards (towards the opening of the plate) and the concentration maximum nearest the rim of the plate (Figure 2a).

Medium

Ensure that the agar plate has a depth of 4.0 ± 0.5 mm, pH 7.3 ± 0.1 and fulfils quality specifications. The medium and supplements will depend on the organism groups being tested (Table 2).

Inoculum preparation

Use the inoculum guide in Table 2. Emulsify several well-isolated colonies from an overnight agar plate in a suitable suspension medium to achieve the specified inoculum turbidity by comparing to a McFarland turbidity standard. For fastidious organisms such as pneumococci, streptococci, gonococci, anaerobes and *Haemophilus* spp., use the suspension prepared in broth within 15 minutes.

Inoculation

Soak a sterile, non-toxic or not too tightly spun swab in the inoculum suspension and remove excess fluid by pressing it against the inside wall of the test tube. Remove more fluid when streaking a 90 mm plate and less for a 150 mm plate. Carefully streak the entire agar surface three times, rotating the plate 60 degrees each time to evenly distribute the inoculum. Alternatively, use Retco C80 (rota-plater, bioMérieux SA) to efficiently streak the inoculum over the agar surface. Allow excess moisture to be absorbed for approximately 15 to 20 minutes so that the surface is completely dry before applying the Etest gradient strips.

Notes:

1. When the inoculum and inoculation are optimal, an even confluent growth will be obtained.
2. McFarland turbidity standards do not guarantee correct number of viable cells in the suspension. Perform colony counts regularly to verify that the inoculum procedure gives the correct number of viable cells in CFU/mL. Please refer to the QUALITY CONTROL section.
Table 2. Recommended media, inoculum and incubation

<table>
<thead>
<tr>
<th>Organism group</th>
<th>Agar media 1)</th>
<th>Inoculum</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobes</td>
<td>Mueller Hinton + 2% NaCl (Etest Oxaclarin only)</td>
<td>0.85% NaCl (or VITEK® 2 Saline 0.45%)10</td>
<td>0.5 (if mucoid)</td>
</tr>
<tr>
<td>ORSA/ ORSE</td>
<td>Mueller Hinton broth (or Brain Heart Infusion broth (BHI))</td>
<td>0.5 (if mucoid)</td>
<td>35 °C</td>
</tr>
<tr>
<td>Anaerobes</td>
<td>Brucella Blood</td>
<td>Brucella broth or Mueller Hinton broth (or Schaedler Broth + vit. K3)10</td>
<td>1</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>HTM (CLSI) MHH (EUCAST)</td>
<td>Mueller Hinton broth or HTM broth (or Brain Heart Infusion broth (BHI))10</td>
<td>0.5 (if mucoid)</td>
</tr>
<tr>
<td>Streptococcus pneumoniae and Streptococci 2)</td>
<td>Mueller Hinton broth (or VITEK 2 Saline 0.45% or BHI broth)10</td>
<td>0.5 (if mucoid)</td>
<td>35 °C</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>GC-agar base + defined supplements</td>
<td>Mueller Hinton broth (or BHI broth)10</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Notes:
1. Please consult Etest documents available at www.biomerieux.com/techlib for further information on specific applications.
2. Includes β-haemolytic Streptococci groups A, B, C and G and viridans group S. mutans, S. mitis, S. sanguis and S. bovis.
3. Use well defined and high quality medium that supports good growth. The brand chosen should have good batch-to-batch reproducibility to ensure that accurate and reliable MIC values are obtained.
4. For trimethoprim and trimethoprim/sulfamethoxazole, ensure that the brand and batch of agar has a low thymine/thymidine content to minimise antagonism of the activity of trimethoprim and sulfonamides.
5. The inherent calcium content in Mueller Hinton agar may vary between brands and batch to batch. Perform quality control of agar plates on a batch-to-batch basis to qualify it for use, particularly for testing of daptomycin.
6. The inherent manganese content in Mueller Hinton agar may vary between brands and batch to batch. Perform quality control of agar plates on a batch-to-batch basis to qualify it for use, particularly for testing of tigecycline.
7. Ensure that an efficient anaerobic system is used to achieve rapid anaerobiosis to avoid false resistant results with metronidazole.
8. When incubating fastidious organisms in 5% CO2, the resulting pH decrease can affect the activity of macrolides, lincosamides, streptogramins, aminoglycosides, quinolones, penicillins and tetracyclines. Please be aware that differences in results can be obtained between systems that are incubated ambiently and in CO2.
9. Ensure the agar plate is incubated for the recommended period before reading, especially for delayed expression of resistance and slow growing and fastidious organisms.
10. VITEK 2 Saline 0.45% (bioMérieux Ref. V1204), Schaedler Broth + vit. K3 (bioMérieux Ref. 42106) and Brain Heart Infusion broth (BHI) (bioMérieux Ref. 42081) have been shown to be compatible with Etest.

INTERPRETATION OF RESULTS

Reading the MIC
After the required incubation period (Table 2), and only when an even lawn of growth is distinctly visible, read the MIC value where the pointed end of the inhibition ellipse intersects the side of the strip. Do not read the plate if the culture appears mixed or if the lawn of growth is too light or too heavy; repeat the test.

Etest MIC endpoints are usually clear-cut although different growth/ inhibition patterns may be seen. Please consult the guidelines below and illustrations in the ETEST READING GUIDE (Figures 7 to 26).

IMPORTANT READING OBSERVATIONS
• Consult the Etest Customer Information Sheet (CIS 006) for information on the mode of action of each antibiotic (bactericidal or bacteriostatic).
• For bactericidal drugs e.g. β-lactams, always read the MIC at the point of complete inhibition of all growth, including hazes, microcolonies and isolated colonies. Tilt the plate and/or use a magnifying glass to carefully examine endpoints, especially for pneumococci, streptococci, enterococci, fusobacteria, Acinetobacter and Stenotrophomonas spp.
• For bacteriostatic drugs e.g. trimethoprim/sulfamethoxazole, in case of trailing endpoints, read at 80% inhibition, i.e. the first point of significant inhibition as judged by the naked eye.

• Excessively wet plates prior to inoculation, insufficient drying before applying strips and/or unevenly streaked surfaces may give non-confluent growth, jagged ellipse edges and uneven MIC intersections. Repeat the test if MIC endpoints are difficult to read.
• When macrocolonies are present within the ellipse for bactericidal agents, read all macrocolonies within 1-3 mm from the strip (consult ETEST READING GUIDE, Figure 21).
• When growth occurs along the entire strip i.e. no inhibition ellipse is seen, report the MIC as ≥ the highest value on the MIC scale. When the inhibition ellipse is below the strip (does not intersect the strip), report the MIC < the lowest value on the MIC scale.
• Organisms such as staphylococci, Acinetobacter spp., anaerobes and gonococci may be susceptible to sulfactam, tazobactam or clavulanic acid per se. For Etest Ptc and TLC, this may result in an inhibition ellipse with an extended parallel band of inhibition alongside the strip. Extrapolate the upper elliptical curvature towards the strip to obtain the MIC (consult ETEST READING GUIDE, Figure 15).
• If inhibition ellipses for clindamycin, erythromycin or chloramphenicol "dip" at the endpoint, extrapolate the MIC at the initial indentation, i.e. 0.5-1 dilution above the intersection.
• For fosfomycin showing numerous (>5 ) macrocolonies in the inhibition ellipse, read the MIC at complete inhibition. A few (≤5) colonies can be ignored.
• For quinupristin/dalfopristin and linezolid, hazy and trailing growth for streptococci, enterococci, fusobacteria, Acinetobacter and Stenotrophomonas spp. when growth occurs along the entire strip i.e. no inhibition ellipse is seen, report the MIC as ≥ the highest value on the MIC scale.
• Vancomycin inhibition ellipses can be slim. Read the actual intersection at the strip and not growth "hugging" the side of the strip.

Interpretation
MIC breakpoints for defining interpretive categories as published by the CLSI®, EUCAST and/or your national reference group may be used for interpreting Etest MIC values.

Being a fully quantitative MIC method, Etest enables the laboratory to report the exact MIC value together with the interpretive category. Etest generates MIC values from a continuous scale and can give results in-between conventional two-fold dilutions i.e. half dilutions. An Etest MIC value which falls between standard two-fold dilutions must be rounded up to the next upper two-fold value before categorisation.

Example: Benzylpenicillin MIC (µg/mL) breakpoints for Streptococcus pneumoniae are:

<table>
<thead>
<tr>
<th>S</th>
<th>I</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 0.06</td>
<td>0.12-1</td>
<td>≥ 2</td>
</tr>
</tbody>
</table>

An Etest MIC of 1 µg/mL is reported as intermediate (I) while 1.5 is rounded up to 2 µg/mL and the category reported as resistant (R).
WASTE DISPOSAL
Unused reagents may be considered as non hazardous waste and disposed of accordingly.
Dispose of used reagents as well as any other contaminated disposable materials following procedures for infectious or potentially infectious products.
It is the responsibility of each laboratory to handle waste and effluents produced according to their nature and degree of hazardousness and to treat and dispose of them (or have them treated and disposed of) in accordance with any applicable regulations.

REFERENCES

Note:
Extensive Etest references based on peer reviewed literature are available from PubMed (internet).

BIBLIOGRAPHY
2. Lorian, V. Antibiotics in Laboratory Medicine. 5th Ed. 2005. Williams & Wilkins, USA.
ORGANISM RELATED EFFECTS

Figure 7.
Ignore swarming. MIC 0.064 µg/mL

Figure 8.
Ignore haemolysis; read the inhibition of growth. MIC 0.032 µg/mL

Figure 9.
Tilt plate or use a magnifying glass to see pin-point colonies and hazes, e.g. enterococci, pneumococci, fusobacteria, and Stenotrophomonas spp. MIC 1 µg/mL

Figure 10.
Scrutinise β-lactam endpoints for pneumococci for hazes and microcolonies. MIC 2 µg/mL

Figure 11.
Bactericidal agents give sharp MIC endpoints. MIC 0.064 µg/mL

Figure 12.
Bactericidal agents; read at complete inhibition of hazes and microcolonies. MIC 1.5 µg/mL

Figure 13.
Bacteriostatic agents; read at 80% inhibition. MIC 1.5 µg/mL

Figure 14.
Linezolid; read at 90% inhibition (ignore finer hazes and pinpoint colonies). MIC 0.75 µg/mL

Figure 15.
β-lactamase inhibitors e.g. tazobactam; extrapolate the upper curvature to the strip. MIC 3 µg/mL

Figure 16.
Trim/sulfa; read at 80% inhibition (ignore lawn of haze within the ellipse). Stenotrophomonas spp. MIC 0.19 µg/mL

Figure 17.
Tigecycline; read at 80% inhibition (ignore trailing microcolonies or hazes). MIC 0.25 µg/mL

Figure 18.
Polypeptides; read at the bottom of the “dip” if colonies are absent. MIC 0.38 µg/mL

DRUG RELATED EFFECTS

Figure 8.
Ignore haemolysis; read the inhibition of growth. MIC 0.032 µg/mL

Figure 10.
Scrutinise β-lactam endpoints for pneumococci for hazes and microcolonies. MIC 2 µg/mL

Figure 11.
Bactericidal agents give sharp MIC endpoints. MIC 0.064 µg/mL

Figure 12.
Bactericidal agents; read at complete inhibition of hazes and microcolonies. MIC 1.5 µg/mL

Figure 13.
Bacteriostatic agents; read at 80% inhibition. MIC 1.5 µg/mL

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Linezolid; read at 90% inhibition (ignore finer hazes and pinpoint colonies). MIC 0.75 µg/mL

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Figure 18.
Polypeptides; read at the bottom of the “dip” if colonies are absent. MIC 0.38 µg/mL
**RESISTANCE MECHANISM RELATED EFFECTS**

- **Figure 19.** Small colony variants and bactericidal agents; read at complete inhibition. MIC 32 µg/mL.
- **Figure 20.** Isolated colonies for oxacillin represent heteroresistant subpopulations i.e. ORSA. MIC 48 µg/mL.
- **Figure 21.** Isolated colonies for carbapenems may represent resistant subpopulations e.g. KPC. MIC 8 µg/mL.
- **Figure 22.** Trailing growth (hazes, microcolonies, macrocolonies) represent VISA/hVISA. MIC 8 µg/mL.

**TECHNICAL AND HANDLING EFFECTS**

- **Figure 23.** Intersection in-between markings, read the upper value. MIC 0.19 µg/mL.
- **Figure 24.** Uneven intersections; read the higher value. If >1 dilution, repeat the test. MIC 0.5 µg/mL.
- **Figure 25.** Ignore a thin line of growth alongside the strip. MIC 0.25 µg/mL.
- **Figure 26.** Complete growth around the strip. MIC ≥ 256 µg/mL.

**WARRANTY AND DISCLAIMER**

**EXPRESS LIMITED WARRANTY AND DISCLAIMER**

BioMérieux SA expressly warrants that Etest will determine the MIC of the antimicrobial agent on each test strip, if the procedures, precautions and important observations indicated in the package insert are strictly complied with.

BioMérieux SA makes no other warranties, expressed or implied, including the implied warranty of merchantability or fitness for particular purpose.

Any change or modification of the product instructions may affect results. BioMérieux SA shall not be liable for any damages resulting from product tampering, variance in transportation, stated storage, handling, testing procedures, precautions and other instructions of the most recently revised version of the package insert.

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Photos: bioMérieux SA

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**INDEX OF SYMBOLS**

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<th>Symbol</th>
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<tr>
<td>REF</td>
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<tr>
<td>IVD</td>
<td>In Vitro Diagnostic Medical Device</td>
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<tr>
<td></td>
<td>Manufacturer</td>
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<td></td>
<td>Temperature limitation</td>
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<td>Upper limit of temperature</td>
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<td>Batch code</td>
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<td>Consult Instructions for Use</td>
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<td>Contains sufficient for &lt;n&gt; tests</td>
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**Etest® Antimicrobial Susceptibility Testing**

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