

Etest®

Antifungal Susceptibility Testing
For In Vitro Diagnostic Use

INTENDED USE

Etest® is an agar-based gradient technique for quantitative antifungal susceptibility testing of *Candida* species. A predefined concentration gradient of a specific antifungal agent is used to determine the Minimum Inhibitory Concentration (MIC) in µg/mL that inhibits the growth of the test organism under defined testing conditions.

PRINCIPLES OF USE

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

The concentration gradient *per se* is a format that is considered useful for detection of various resistance mechanisms. Studies have shown, for example, that Etest can efficiently detect hetero-resistance and intrinsic resistance to azoles in *Candida krusei* and *C. glabrata*.

Although processed like the disc diffusion test, the preformed and stable antifungal concentration gradient in Etest clearly differentiates the two methods. Unlike disc diffusion, Etest MIC results are not similarly affected by the physico-chemical properties of the drug e.g. molecular weight, solubility and diffusion coefficient nor the varying growth characteristics of different organisms.

Etest consists of a thin non-porous rectangular plastic strip. One side of the strip (A) carries a letter code designating the identity of the antifungal agent and is calibrated in terms of MIC values in µg/mL. A predefined and exponential gradient of the dried and stabilised antifungal agent is immobilised on the opposite surface (B) of the strip 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.

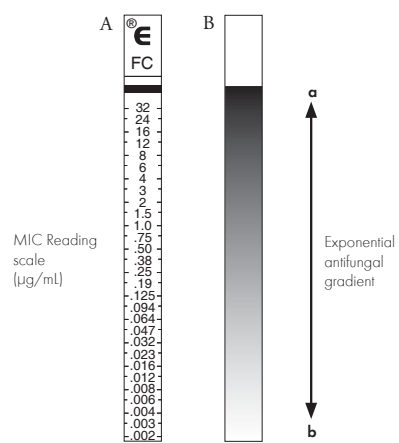


Figure 1: Etest gradient configuration

When the Etest strip is applied onto an inoculated agar surface, the antifungal agent is immediately released from the plastic surface into the agar matrix. A stable, predefined and continuous concentration gradient of the antifungal agent is established and maintained underneath and alongside the strip. After incubation, whereby growth becomes visible, a symmetrical inhibition ellipse centred along the strip will be seen. The inhibition ellipse intersects the strip at the MIC value in µg/mL. When different growth-inhibition patterns are seen, the MIC endpoint should be selected according to criteria described under **INTERPRETATION** and as illustrated in the **ANTIFUNGAL READING GUIDE**.

REAGENT

Etest is supplied in a package of 100 or 30 test strips of one antifungal agent.

STORAGE

All packages must be stored either at controlled room temperature (18-22 °C) or freezer at (-18-22 °C) as specified on the product label, until the

given expiry date.

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 colour-indicating desiccant, and stored at the temperature stated on the label or at -20 °C. 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.

Prevent moisture from penetrating into or forming within the package or storage containers. Keep Etest strips dry.

HANDLING

Remove the original package or storage containers with Etest strips from the freezer and allow them to reach room temperature before opening (-20 °C/ approximately 60 minutes). Ensure that moisture condensing on the outer surface has evaporated completely before opening the package or storage container. Packages stored at controlled room temperature can be used immediately.

Open the package by carefully cutting off the top of one blister partition, or across the top of the foil pouch. When handling Etest strips manually, grip only the handle of the strip i.e. the area labelled E. **Do not touch the surface of the strip with the antifungal gradient, i.e. the side opposite the MIC scale** (Figure 1). Place the strips in an Etest applicator tray or a dry Petri dish until ready for use. The vacuum pen Nema C88™ (AB BIODISK) can be used to efficiently apply Etest antifungal strips from the applicator tray or the original foam cartridge. The foam cartridges carrying Etest strips should be loaded directly onto the automatic applicator instrument Simplex C76™ (AB BIODISK).

PRECAUTIONS AND WARNINGS

- Etest is intended for *in vitro* diagnostic use only.
- Although based on a simple procedure, only personnel trained in mycology and antifungal susceptibility testing techniques should use Etest.
- Etest should be used strictly according to the procedures described in this product insert.
- Aseptic procedures should be observed at all times when handling yeast specimens and established precautions against microbiological hazards strictly followed. Agar plates should be sterilised after use and before discarding.

PROCEDURE

Materials provided

- 100 or 30 Etest gradient strips of one antifungal agent
- 1 desiccant capsule
- Package insert
- Table 1

Materials required but not provided

- RPMI agar plates (depth 4 ± 0.5 mm)
- Sterile saline (0.85% NaCl)
- Sterile loops, swabs, test tubes, vortex mixer and scissors
- Forceps, Etest manual applicator or Biotools™ (Retro C80™, Nema C88, Simplex C76)
- 0.5 and 1 McFarland turbidity standards
- Incubator (35 ± 2 °C)
- Quality control strains
- Storage containers with desiccant capsules, or pouches and/or sealing clamps
- Additional technical information: www.abbiotest.com/Etest Technical Manual

Medium

RPMI 1640 broth (with L-glutamine and phenol red without bicarbonate) + MOPS (0.165 mol/L) + 2% glucose + 1.5% Bacto agar (pH 7.0 ± 0.1) should be used. When using RPMI 1640 media from different commercial sources, quality control (QC) should always be performed using the specified reference strains to ensure that MIC results obtained are within QC specifications (see **QUALITY CONTROL**). Do not use a particular media brand or batch if results are not within the QC limits. More technical information on the preparation of RPMI agar plates can be obtained at www.abbiotest.com.

Inoculum preparation

Homogenise several well-isolated colonies from a 24 to 48 hour pure culture on Sabouraud dextrose agar in saline to obtain a turbidity equivalent to 0.5 McFarland standard. For mucoid organisms, use a 1 McFarland standard to compensate for turbidity associated with the strain's capsular material.

Inoculation

Soak a sterile, non-toxic swab (soft and not too tightly spun) into the inoculum suspension and press the swab against the inner wall of the test tube to remove excess fluid. Carefully streak the entire agar surface in three directions to evenly distribute the inoculum. **Soak the swab again and repeat the streaking procedure a second time.** Alternatively, the

agar plate can be evenly inoculated using Retro C80™ (rota-plater, AB BIODISK) by streaking the surface and then soaking the swab again before streaking a second time. Allow excess moisture to be fully absorbed for approximately 15 to 20 minutes so that the **agar surface is completely dry before applying the Etest strips.**

Notes

- If the inoculum density and inoculation are correct, an even lawn of confluent growth will be obtained after incubation (see **QUALITY CONTROL**).
- McFarland turbidity standards do not guarantee correct inoculum in terms of CFU/mL. Perform regular colony counts to verify the inoculum density.

Application

A template can be used to optimally position several strips in an equidistant pattern on a 150 mm agar plate or 1 to 2 strips on a 90 mm agar plate (Figures 2a and 2b). Apply the strip to the agar surface with the **MIC scale facing upwards** i.e. towards the opening of the plate. Do not place the strip upside down as the test will not work because the antifungal agent cannot diffuse across the non-porous plastic carrier.



Figure 2a: Applying 6 Etest strips to a 150mm plate

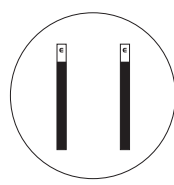


Figure 2b: Template for 2 Etest strips per 90mm plate

Etest strips can be applied with forceps, a manual Etest applicator, Nema C88 or Simplex C76. **Position the strip with the concentration maximum towards the rim of the plate** (Figure 2a). Ensure that the whole length of the strip is in complete contact with the agar surface. If large air pockets are seen underneath the strip, remove them by gently pressing on the strip (without moving it) with the applicator tip or forceps, moving from the lowest concentration upwards. Small bubbles under the strip will not affect results. **Once applied, do not move the strip as the antifungal agent is instantaneously released into the agar.**

Incubation

Incubate the inverted agar plates in an ambient and moist atmosphere in an incubator at 35 °C until growth is clearly seen. To maintain moisture, agar plates can be placed in a loosely folded plastic bag before placing in the incubator. Most *Candida* species will grow well within 24 hours although the confirmatory reading should be done at 48 hours. This will accommodate slower growing *Candida* species and ensure that delayed expression of hetero-resistance is detected.

INTERPRETATION

Reading the MIC

After the required period of incubation and when the lawn of growth is clearly visible, read the MIC value where the inhibition ellipse intersects 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.

When different growth-inhibition patterns and trailing endpoints are observed, please consult the important reading observations below and illustrations in the **ANTIFUNGAL READING GUIDE** for correct interpretation of MIC endpoints.

IMPORTANT READING OBSERVATIONS

- The yeast species, antifungal agent, medium, incubation period, inoculum and resistance mechanism can affect the appearance of the MIC endpoint, especially for azoles.
- For flucytosine, read trailing endpoints at approximately 90% inhibition of growth, ignoring faint hazes and minute colonies.
- Azoles may give diffuse endpoints, more so for some *Candida* species than others. Read the MIC at the **first point of significant inhibition or marked decrease in growth density**. Use the principle of the so-called 80% inhibition to visually select the MIC endpoint.
- Amphotericin B endpoints should be read at 100% inhibition, i.e. at complete inhibition of growth including all microcolonies, hazes and isolated colonies.
- For echinocandins (e.g. caspofungin), read trailing endpoints at the first visual point of significant inhibition i.e. 80% inhibition.
- The **inoculum density** may affect the clarity of the endpoint, especially for azoles. A lighter but correct inoculum generally gives clearer results.
- The **inoculation technique** may affect the ap-

pearance of the endpoint. Streak evenly in three directions or use the Retro C80 to optimise inoculation. Perform the streaking twice (**dipping in the inoculum suspension in-between streaking**) to ensure complete and even coverage of the agar surface.

- Excessively wet plates and/or unevenly streaked surfaces may give non-confluent growth, jagged ellipse edges and uneven MIC intersections. Repeat the test if endpoints are uneven or difficult to read.
- The RPMI media brand and batch may affect results:
 - RPMI 1640 as recommended by CLSI M27-A2 standard supports growth of most *Candida* species with the exception of some isolates of *C. glabrata*, *C. lusitanae*, *C. krusei* and *C. parapsilosis*. Weaker growth can potentially result in false susceptibility, thus confirmation after 36 to 48 hour incubation is recommended, especially when overnight growth is weak.
 - Use a well defined and high quality RPMI 1640 medium with glucose to support good growth and minimise excessive trailing endpoints for azoles. The brand of media chosen should also have good batch-to-batch reproducibility so that accurate and reliable MIC values can be obtained.
- The incubation period can affect the clarity of endpoints, especially for azoles. When testing *Candida* species with azoles, plates showing good growth should preferably be read after 18 to 24 hours and confirmed at 36 to 48 hours.
- When growth occurs along the entire strip i.e. no inhibition ellipse is seen, the MIC should be reported as greater than or equal to (≥) the highest value on the MIC scale. When the inhibition ellipse is below the strip i.e. the ellipse does not intersect the strip, the MIC should be reported as less than the lowest value on the MIC scale.

Interpretation

Being a fully quantitative method, Etest provides on-scale results and allows the laboratory to report the specific MIC value as well as the interpretive category. Etest also generates MIC values from a continuous concentration gradient and can give numerical results that fall between conventional two-fold dilution values. An Etest MIC value that is between a two-fold dilution must be rounded up to the next upper two-fold value before interpretive categorisation. For example, an Etest fluconazole MIC value of 48 µg/mL is first rounded up to 64 µg/mL, and the category reported as resistant (R ≥ 64), according to interpretive criteria in **TABLE 1** (separate enclosure).

QUALITY CONTROL

In order to check the quality and performance of Etest reagents, agar media, methods and the correct selection of MIC endpoints, quality control reference strains shown in Table 1 should be tested as described under **PROCEDURE**. The reagents and test procedure can be considered satisfactory only when Etest MIC values for the reference strains fall within the specified quality control limits.

Etest quality control ranges may not be identical to CLSI specifications in all cases. Etest ranges at 48 hours are based on extensive data generated from quality control testing of a large number of reagent lots over several years and also include data from multi-site studies.

Regular colony counts should also be performed during QC to verify that the inoculum is correct in terms of CFU/mL. For example, dilute the inoculum suspension 1:1000 and subculture 10 µL onto a Sabouraud dextrose agar plate and incubate for 36 to 48 hours. An acceptable inoculum for Etest should give approximately 10 to 50 colonies per plate i.e. 1 - 5 x 10⁶ CFU/mL.

Patient results should not be reported if quality control results are outside the stated ranges. The individual laboratory should establish the frequency of their quality control testing. Guidelines provided in CLSI M27-A2 standard are recommended.

PERFORMANCE

Etest performance has been established using comparative evaluations at three or more clinical sites and in-house testing. These investigations compared Etest to the reference CLSI broth microdilution procedure as described in the CLSI M27-A2 standard. Agreement within two dilutions was used to assess the essential agreement (EA) of MIC values obtained with Etest and the reference method. This is accepted praxis in antifungal testing evaluations in order to accommodate the general difficulty in reading antifungal MIC endpoints.

Performance data are summarised in Table 1. Etest 48 hour results showed very good correlation to the reference method read at 48 hours. For some strains, itraconazole resistance as defined by the reference method at 48 hours was better detected at 48 hours

by Etest.

LIMITATIONS

- Antifungal susceptibility testing with all methods, including Etest, requires experience and should be performed only by personnel trained in mycology and susceptibility testing.
- Antifungal susceptibility testing by all methods, including Etest, should only be performed with isolated pure colonies.
- As azoles can have various types of trailing endpoints, judgement of the MIC may be difficult, particularly for inexperienced personnel. Users should practice reading the different types of endpoints and compare their performance with trained personnel to improve proficiency and achieve consistency in selecting the correct Etest MIC endpoints.
- Certain differences between Etest as an agar-based gradient method, and broth dilution procedures based on other technical principles may occur due to characteristics inherent in these test formats. An example could be more efficient detection of intrinsic azole resistance by Etest in *C. krusei* and *C. glabrata* species.
- As with all methods for antifungal susceptibility testing, the results obtained with Etest are *in vitro* values only and may provide an indication of the organism's *in vivo* susceptibility. **The final interpretation of the MIC result to guide therapy selection must be the sole decision and responsibility of the attending physician, who should base judgement on the particular history and knowledge of the patient, the pharmacology and pharmacokinetics of the antifungal agent and prior clinical experience in treating infections caused by the particular species of yeast pathogen with the antifungal agent being considered.**

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isolates of *Candida* species determined by broth microdilution, disk diffusion and Etest methods: Report from Artemis global antifungal susceptibility program 2001. *JCM*. 41(4): 1440-1446.

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12. Laverdiere M., Restieri C., Habel F. (2002). Evaluation of the *in vitro* activity of caspofungin against bloodstream isolates of *Candida* species from cancer patients: comparison of Etest and NCCLS reference methods. *International Journal of Antimicrobial Agents*. 20(6): 468-471.

13. Etest references on antifungal susceptibility testing – data on file available via www.abbioidisk.com or PubMed search.

ETEST ANTIFUNGAL READING GUIDE

AZOLES

When trailing endpoints occur, read the MIC at the first point of significant inhibition of growth i.e. the so-called 80% inhibition as judged by the naked eye. When trailing is strong, especially with species such as *C. glabrata* with reduced azole susceptibility, and for smaller ellipses at higher MIC values, read the plate against a strong light source to facilitate visual detection of the endpoint at 80% inhibition.



Figure 1:
C. albicans,
clear endpoint
MIC: 0.047 µg/mL

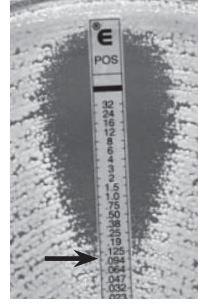


Figure 2:
Candida spp.,
slim ellipse with
trailing colonies
MIC: 0.094 µg/mL

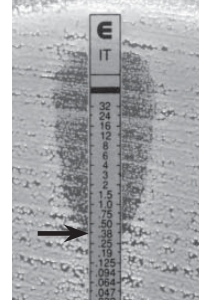


Figure 3:
C. tropicalis,
discernable ellipse
with a lawn of
microcolonies
MIC: 0.38 µg/mL

AMPHOTERICIN B

Read endpoint at complete inhibition of growth i.e. 100% inhibition including all microcolonies, hazes and isolated colonies.



Figure 10:
Candida spp.,
clear endpoint
MIC: 0.094 µg/mL

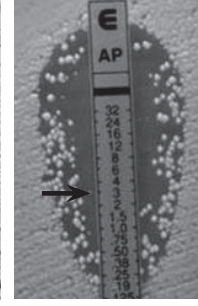


Figure 11:
Candida spp.,
macrocolonies in the
ellipse
MIC: 3 µg/mL

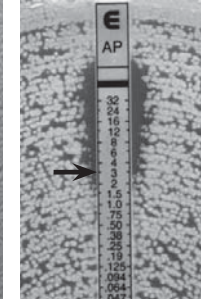


Figure 12:
Candida spp.,
slim ellipse and
microcolonies
MIC: 3 µg/mL

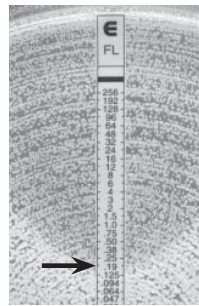


Figure 4:
C. albicans,
discernable ellipse
with a lawn of
microcolonies
MIC: 0.19 µg/mL



Figure 5:
C. parapsilosis,
trailing colonies
MIC: 0.25 µg/mL

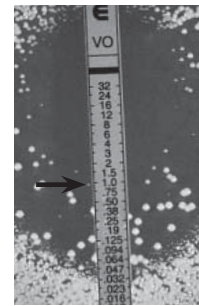


Figure 6:
Candida spp.,
macrocolonies in the
ellipse, (potential
hetero-resistance)
MIC: 1 µg/mL

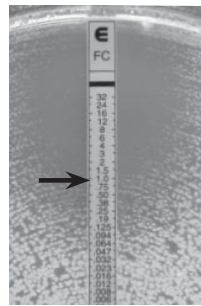


Figure 13:
C. tropicalis,
trailing colonies
MIC: 1 µg/mL

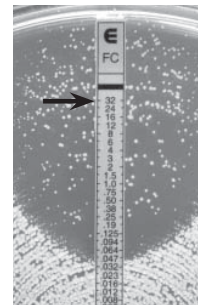


Figure 14:
C. albicans,
macrocolonies in the
ellipse
MIC: ≥ 32 µg/mL

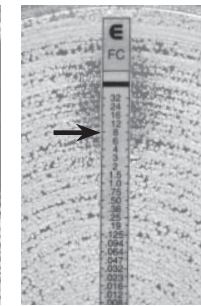


Figure 15:
C. albicans,
higher MIC with
trailing colonies
MIC: 8 µg/mL

FLUCYTOSINE

When trailing endpoints occur, read the MIC at approximately 90% inhibition, as judged by the naked eye. Ignore faint hazes and minute microcolonies.

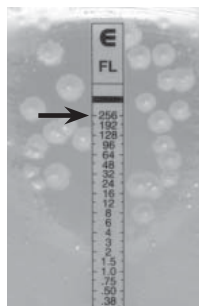


Figure 7:
C. krusei,
macrocolonies in the
ellipse
MIC: ≥ 256 µg/mL

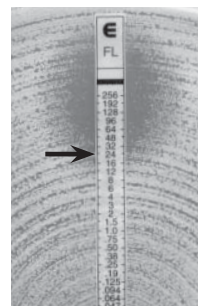


Figure 8:
C. glabrata,
higher MIC with
trailing colonies
MIC: 24 µg/mL

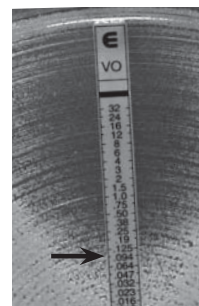


Figure 9:
Candida spp.,
trailing colonies
MIC: 0.094 µg/mL

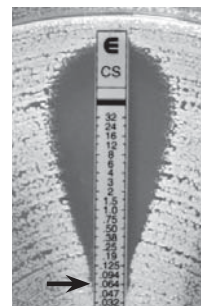


Figure 16:
C. albicans,
"dip effect" - read the
MIC at the bottom of
the dip
MIC: 0.064 µg/mL

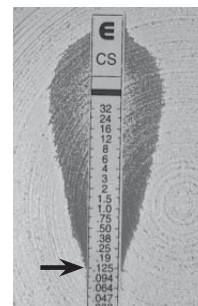


Figure 17:
C. tropicalis,
ellipse with re-
growth at the higher
concentration range
- ignore re-growth
MIC: 0.125 µg/mL

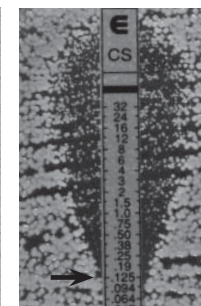


Figure 18:
Candida spp.,
discernable ellipse
with microcolonies
MIC: 0.125 µg/mL

WARRANTY AND DISCLAIMER

EXPRESS LIMITED WARRANTY AND DISCLAIMER

AB BIODISK expressly warrants that Etest will determine the MIC of the antifungal agent on each test strip, if the procedures, precautions and limitations indicated in this package insert are strictly complied with. If the test strip does not do so, AB BIODISK shall refund the cost of the product or replace the defective test strips.

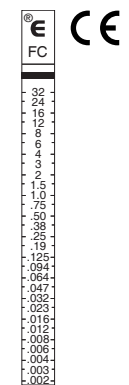
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Etest®
Antifungal Susceptibility Testing
For *In Vitro* Diagnostic Use

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CASPOFUNGIN (ECHINOCANDINS)

When trailing endpoints or other growth/inhibition phenomena occur, read the MIC at the first visual point of significant inhibition i.e. 80% inhibition.

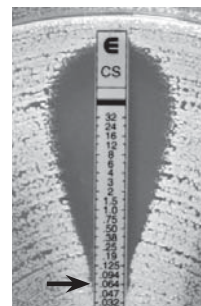


Figure 16:
C. albicans,
"dip effect" - read the
MIC at the bottom of
the dip
MIC: 0.064 µg/mL

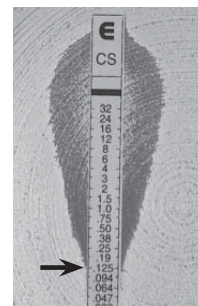


Figure 17:
C. tropicalis,
ellipse with re-
growth at the higher
concentration range
- ignore re-growth
MIC: 0.125 µg/mL

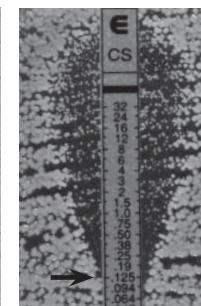


Figure 18:
Candida spp.,
discernable ellipse
with microcolonies
MIC: 0.125 µg/mL

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