

Etest for Antifungal Susceptibility Testing

For *In Vitro* Diagnostic Use

In the USA, For Investigational Use Only

BACKGROUND

Invasive fungal infections have increased over the past two decades causing formidable morbidity and mortality among immunocompromised hosts, especially patients with AIDS. The frequency of nosocomial fungal infections has also increased amongst cancer, organ transplant, burn and surgical patients. Due to the life threatening nature of these infections and reports of drug resistance, susceptibility testing of yeast pathogens has become very important.

Methods used for susceptibility testing of yeasts include disc diffusion, agar dilution and broth dilution procedures. Numerous *in vitro* factors such as media, buffer, inoculum, incubation and end point criteria can affect results significantly. Most methods are therefore reported to provide at best an estimation of the MIC value within 3 two-fold dilution steps.

The NCCLS have published a reference method for broth dilution antifungal susceptibility testing of yeast (M27-A, June, 1997). A broth macro- and microdilution format using a defined medium (RPMI 1640 buffered with MOPS) and 48 hour incubation forms the basis for this method. Quality control specifications for two reference strains and interpretive guidelines for *Candida* species are provided for fluconazole, itraconazole and flucytosine. Since the M27 methodology does not consistently detect amphotericin resistance, tentative criteria of MIC >1 µg/ml is likely to suggest resistance.

The NCCLS reference standard can now be used to develop and validate alternative methods that may be more suitable for routine use in the clinical laboratory.

SUSCEPTIBILITY TESTING

Susceptibility testing of yeasts is complicated by variables related to the drug, organism and technique used. The following factors should be considered when designing, performing and interpreting tests with different drug/organism combinations.

Organism specific factors

1. Variable growth characteristics.
2. Pleomorphism i.e. certain fungi grow as a unicellular yeast form and reproduces by budding while others may consist of a hyphal-mold form that may produce asexual spores.
3. Most yeasts are aerobes, although some may grow anaerobically.
4. Medium, pH and incubation temperature can affect growth and pleomorphism.

Drug specific factors

1. Limited aqueous solubility of some agents.
2. Partial inhibition of growth over a wide concentration range giving trailing end points; typically seen with azoles.
3. Buffer and pH effects on activity.
4. Interaction with media components and buffer.

Variables that influence results

1. Inoculum.
2. Medium formulation and pH.
3. Agar versus broth.
4. Type of buffer (MOPS versus phosphate).
5. Temperature and duration of incubation.
6. MIC end point criteria.

ANTIFUNGAL AGENTS

Polyene: Amphotericin B acts by binding to ergosterol in the cell membrane causing loss of membrane integrity and results in osmotic instability. Amphotericin has fungicidal activity and MIC end points are read at complete inhibition of growth. Resistance, though rare, is sometimes seen with *C. lusitaniae* due to changes in membrane composition.

Azoles: Imidazoles such as ketoconazole and triazoles such as fluconazole and itraconazole have a broad spectrum of activity against yeasts and dimorphic fungi. Azoles inhibit fungal cytochrome P450 dependant enzymes, impairing ergosterol synthesis thus depleting ergosterol in the cell membrane. Activity is fungistatic, partial inhibition of fungal growth occurs over a wide concentration range. This results in trailing of growth and MIC end points are read at the so-called 80% inhibition or first point of significant inhibition compared to the growth control. Resistance has been reported among others in *C. albicans*, *C. glabrata* and *C. tropicalis*. *C. krusei* is considered to be intrinsically resistant to azoles.

Flucytosine, a fluorinated pyrimidine, is an antimetabolite for uracil in the synthesis of yeast RNA. *In vitro* activity is antagonized by purines, pyrimidines and nucleosides and synthetic media free of these substances must be used for testing. Intrinsic resistance and resistance development during therapy have been well documented.

INTENDED USE

Etest can be used for antifungal susceptibility testing of yeasts such as *Candida albicans* and other *Candida* spp. and *Cryptococcus neoformans*. It provides the minimum inhibitory concentration (MIC $\mu\text{g/ml}$) of individual antifungal agents. The MIC value of a given agent is defined by the experimental conditions used. Thus, MIC values obtained under different *in vitro* conditions cannot be used interchangeably unless shown to be equivalent to the reference method.

EXPLANATION

Principles of Etest

Etest is based on a combination of the concepts of dilution and diffusion tests. Like dilution methods, Etest directly quantifies antifungal susceptibility in terms of discrete MIC values. As Etest consists of a predefined and continuous concentration gradient, the MIC values obtained can be more precise than values from conventional procedures based on discontinuous two-fold serial dilutions. Although processed like the disc diffusion test, the predefined and stable concentration gradient in Etest, differentiates the two methods clearly. Unlike disc diffusion, Etest MICs are unaffected by drug properties such as molecular weight, aqueous solubility and diffusion characteristics or by varying growth rates of different yeasts.

Etest consists of a thin, inert and non-porous plastic strip (5 x 60 mm). One side of the strip is calibrated with MIC values in $\mu\text{g/ml}$ and a two-letter code designates the identity of the drug. A predefined and exponential gradient of the dried and stabilised antifungal agent is immobilised on the other surface of the strip with the concentration maximum at a, and the minimum at b (figure 1). The continuous gradient covers a concentration range corresponding to 15 two-fold dilutions in a conventional dilution procedure.

When applied onto an inoculated agar plate, there is an immediate release of the agent from the plastic surface into the agar matrix. A predefined, continuous and stable gradient of the drug concentrations is created directly underneath the strip. After incubation, whereby growth becomes visible, a symmetrical inhibition ellipse centred along the strip is seen. The zone edge intersects the strip at the MIC value. When different growing-inhibition patterns are seen, the MIC end point should be selected according to criteria described under Interpretation .

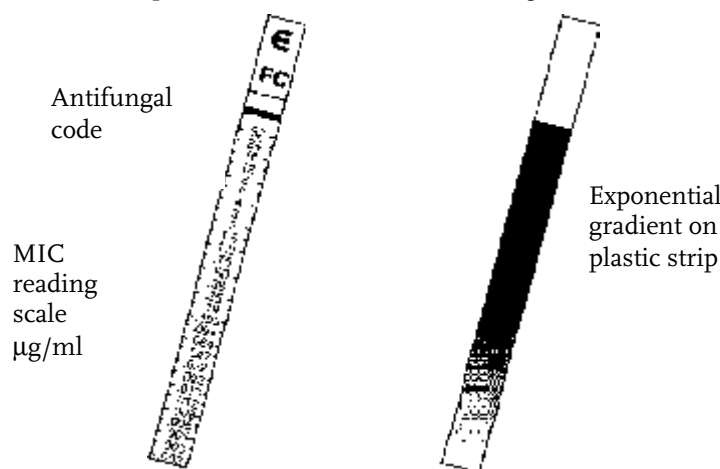


Figure 1

REAGENTS

100 units per pack of the following agents are available:

Agent	Code	MIC range (µg/ml)
Amphotericin B	AP	0.002 - 32
Flucytosine	FC	0.002 - 32
Fluconazole	FL	0.016 - 256
Itraconazole	IT	0.002 - 32
Ketoconazole	KE	0.002 - 32

STORAGE

All unopened packages should be stored at -20°C until the given expiration date.

Unused strips stored at -20°C in an air tight storage tube or container with dessicant can be used until the expiration date.

HANDLING

CARE MUST BE TAKEN TO AVOID MOISTURE PENETRATING INTO OR FORMING WITHIN THE PACKAGE OR STORAGE CONTAINER.

Remove the package or storage container from the freezer (-20°C) and allow to equilibrate to room temperature for approximately 20 minutes. Moisture condensing on the outer surface must be allowed to evaporate, before opening. Inspect the package and storage container for holes or cracks. Do not use the strips if the package has been damaged.

To open the package, cut along the broken lines at the top of the blister compartment: do not cut in between compartments. Remove the strips from the package, gripping the handle of the strip (area labelled E). Separate the strips by twisting them apart. Do not touch the surface of the strip with the antifungal gradient, i.e. the side underneath the MIC scale (figure 1). Place the strips in an Etest Applicator tray or in the lid of a dry petri dish until ready for use as described under Application .

PRECAUTIONS AND WARNINGS

1. Etest is intended for in vitro diagnostic use only.
2. Although a straightforward procedure is used to obtain MICs, proper use of Etest requires the judgement of skilled personnel trained in microbiology and antifungal susceptibility testing techniques.
3. Aseptic procedures should be observed at all times when handling bacterial specimens and established precautions against microbiological hazards strictly adhered to. Agar plates should be sterilized after use, before discarding.

PROCEDURES

Materials provided

100 Etest strips of one agent.

1 package insert.

Materials required but not provided

Agar plates with appropriate media (depth of 4 ± 0.5 mm).

Sterile saline (0.85% NaCl) for inoculum preparation.

Sterile loops, swabs, test tubes, pipettes, forceps, applicator, template and scissors.

McFarland 0.5 and 1 turbidity standards.

Incubator (35°C).

Quality control strains.

Storage containers with dessicant.

Medium ¹⁾

RPMI 1640 broth + MOPS + 2% Glucose + 1.5% agar

Media preparation for 1 litre

RPMI 1640 ²⁾	46.19 g
(contains 0.165M MOPS and L-glutamine)	
Bacto agar	15 g
Glucose	20 g

1. Dissolve the RPMI powder in 500 ml deionized water.
2. Filter sterilize with a 0.2 µm filter.
3. Dissolve the glucose and Bacto agar in 500 ml deionized water. Autoclave for 15 minutes at 15 psi pressure (approx. 121°C) and then cool to approx. 50°C.
4. Gently warm the sterile RPMI + MOPS solution to approximately 45°C and mix it with the cooled glucose-agar solution. Adjust the pH to 7.0.
5. Pour the appropriate volume into sterile petri dishes to achieve an agar depth of 4 ± 0.5 mm after solidification.

Note ¹⁾ RPMI 1640 as recommended by NCCLS for broth dilution procedures supports growth of most *Candida* spp with the exception of some strains of *C. glabrata*, *C. lusitaniae*, *C. krusei* and *C. parapsilosis*. *C. neoformans* do not generally grow well on this medium.

²⁾ The RPMI media specified above contains MOPS and is supplied by Angus Biochemicals (see reference). When using RPMI media from other sources, check the formulation and validate using quality control strains. Batch to batch variations occur and trailing of azole endpoints can be excessive with certain brands.

Inoculum preparation

Homogenize well isolated colonies of *Candida* isolates from an overnight Sabouraud Dextrose agar plate in 0.85% NaCl to achieve 0.5 McFarland turbidity. For *C. neoformans*, use 48-72 hour colonies to achieve a 1 McFarland turbidity.

Inoculation

Dip a sterile, non-toxic swab (not too tightly spun) into the inoculum suspension and press the swab against the inside of the tube. Swab the entire agar surface evenly, rotating 90° in three directions. Allow excess moisture to absorb for about 10-15 minutes so that the agar surface is completely dry before applying the Etest strips.

Notes:

1. If the inoculum density and inoculation procedure is done correctly, a confluent lawn of growth will be obtained after incubation.
2. McFarland standards do not guarantee correct colony counts in cfu/ml of viable cells. Please refer to the QUALITY CONTROL section.

Application

Open the Etest package as described under **Handling**. With a pair of forceps or your fingers, remove the required number of Etest strips and place them on a dry clean surface or in the Etest applicator tray (figure 2a).

Using an Etest applicator or forceps, apply the strips to the inoculated agar surface, ensuring that the MIC scale is facing upwards, i.e. towards the opening of the plate, and that the concentration maximum is nearest the rim of the plate (figure 2b). Make sure the whole length of the strip is in complete contact with the agar surface. If incorrectly placed upside down, no inhibition ellipse will form because the antibiotic will not diffuse across the non-porous plastic strip. If air pockets are seen underneath the strip, remove them by pressing gently on the strip (without moving the strip) with a pair of forceps, always move from the minimum concentration upwards. Small bubbles under the strip will not affect results. Once applied, the strip cannot be moved because of the instantaneous release of drug into the agar.

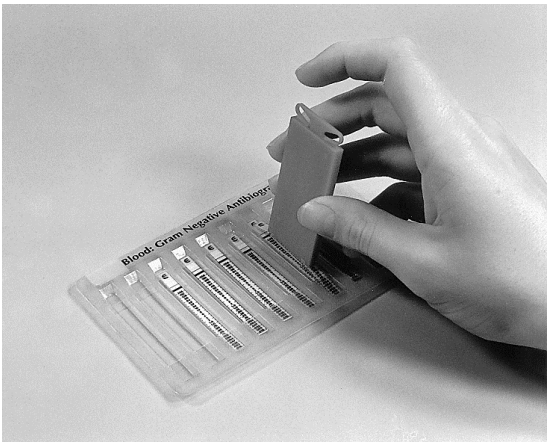


Figure 2a.

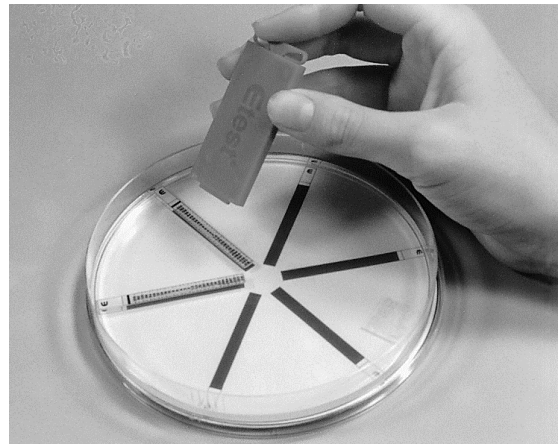


Figure 2b.

Template for application

Use a template for optimal positioning of strips on the agar plate. Four to six different Etest strips can be applied onto a 150 mm agar plate using a template as shown in figure 3a. For single MIC determinations, one to two Etest strips can be used on a 90 mm agar plate (figure 3b).

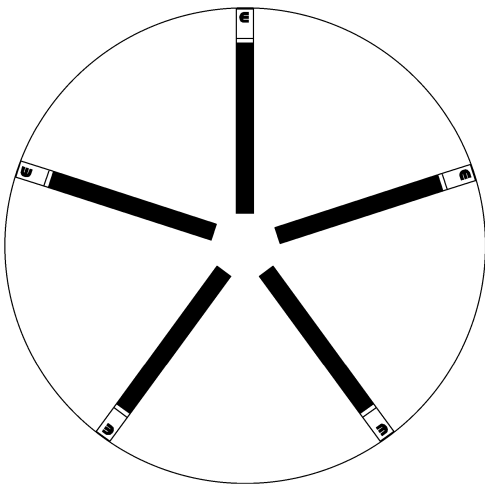


Figure 3a.

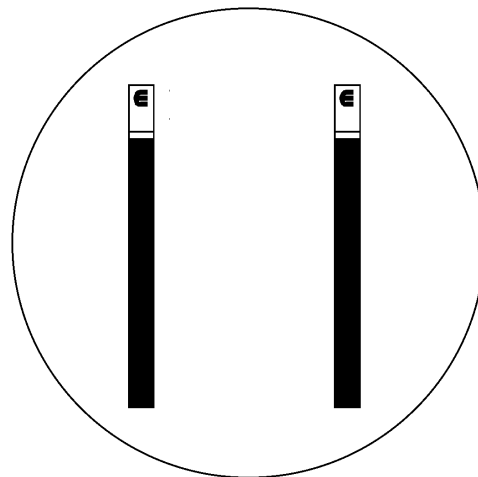


Figure 3b.

Incubation

Incubate agar plates at 35°C for 24 to 48 hours for different *Candida* spp. and 48 to 72 hours for *Cryptococcus neoformans*.

INTERPRETATION OF RESULTS

Reading the MIC

After the required period of incubation whereby growth becomes distinctly visible, read the MIC value at the point of intersection between the zone edge and the Etest strip if the end point is clear. When different growth-inhibition patterns are seen, use the illustrations in the Etest antifungal reading guide to correctly select the MIC end point (figures 4, 5 and 6).

When growth occurs along the entire strip i.e. no inhibition ellipse is seen, the MIC should be reported as > than the highest value on the MIC scale. When the inhibition ellipse is below the strip i.e. the zone edge does not intersect the strip, the MIC should be reported as < than the lowest value on the MIC scale.

Important reading observations

1. The yeast species, antifungal agent, medium, inoculum density and incubation period will affect the clarity of end points, especially for azoles.
2. For flucytosine, read at almost complete inhibition of growth (95%) and for amphotericin B, at the point of complete inhibition (100%).
3. For azoles, read the MIC at the first point of significant inhibition or marked decrease in growth density. Use the principle of 80% inhibition to visually select the end point.

INTERPRETATION

NCCLS M27-A document provides the following interpretive guidelines:

	Susceptible	S-Dose ¹⁾ Dependent	Intermediate ²⁾	Resistant
Fluconazole ³⁾	≤8	16-32	-	≥64
Flucytosine	≤4	-	8-16	≥32
Itraconazole ⁴⁾	≤0.125	0.25-0.5	-	≥1
Amphotericin B	≤0.5 ⁵⁾	-	-	≥2 ⁵⁾

Notes

1. Susceptible-Dose Dependent (S-DD) - dependent on achieving maximum blood level. For fluconazole, doses of ≥400 mg/day dose may be required in adults with normal renal function and body habitus. For itraconazole, steps to assure adequate drug absorption and plasma levels of >0.5 µg/ml may be required for optimal response.
2. The susceptibility of these isolates are uncertain and available data do not permit them to be clearly categorised as susceptible or resistant.
3. Guidelines are based on experience with mucosal infections but are consistent with the limited data available for invasive infections due to *Candida* spp.. *C. krusei* are assumed to be intrinsically resistant to fluconazole and should not be interpreted using these guidelines. The 8 µg/ml upper boundary for the susceptible range is not known with certainty, either 4 or 8 µg/ml may be selected as the cut-off (reference 17c)
4. Guidelines are based entirely on experience with mucosal infections. Breakpoints for invasive infections are not available.
5. MIC distribution (based on NCCLS M27-A) for *Candida* spp. are tightly clustered between 0.25 and 1 µg/ml. For isolates resistant in animal models, M27-A values of >1 µg/ml may be obtained. The M27-A methodology does not consistently detect resistance and presently values of >1 µg/ml suggest that the *Candida* spp. isolate is likely to be resistant”.

ETEST READING GUIDE FOR YEASTS

Flucytosine (read at almost complete inhibition, ignoring small micro-colonies)



Figure 4a. MIC 1 µg/ml.

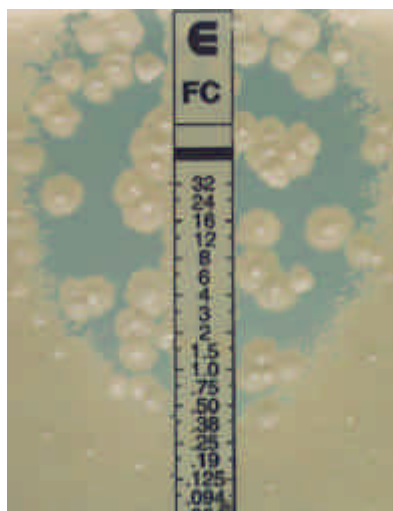


Figure 4b. MIC >32 µg/ml (macrocolonies in ellipse).

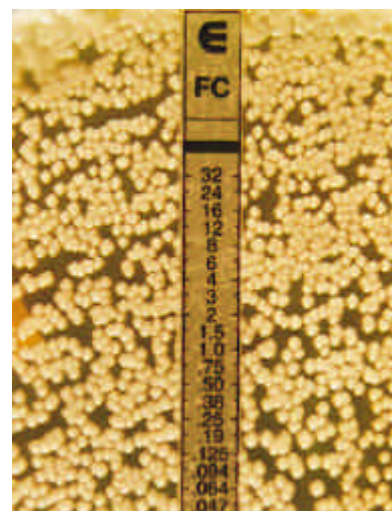


Figure 4c. MIC >32 µg/ml (homogeneously resistant).

Amphotericin B (read at complete inhibition of all growth).

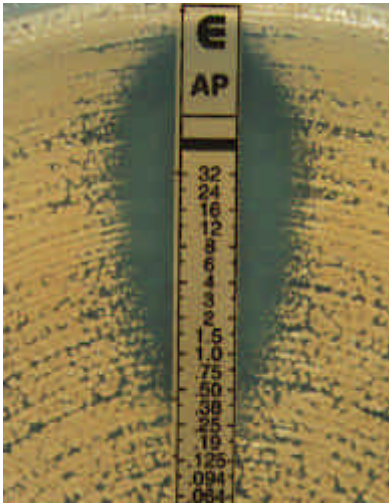


Figure 5a. MIC 1 µg/ml (include all microcolonies).

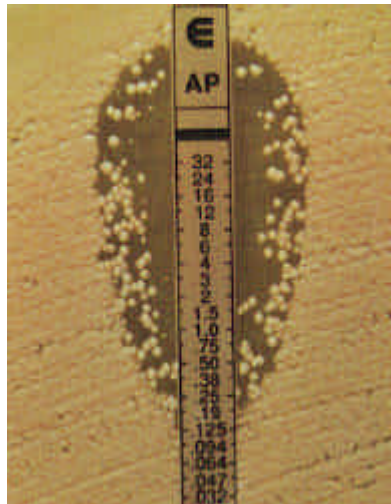


Figure 5b. MIC 3 µg/ml (macrocolonies in ellipse).

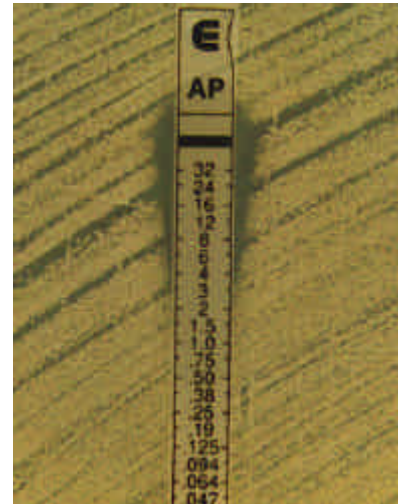


Figure 5c. MIC 4 µg/ml (small and slim ellipse).

Azoles (read at first point of significant inhibition i.e. so-called 80% inhibition).

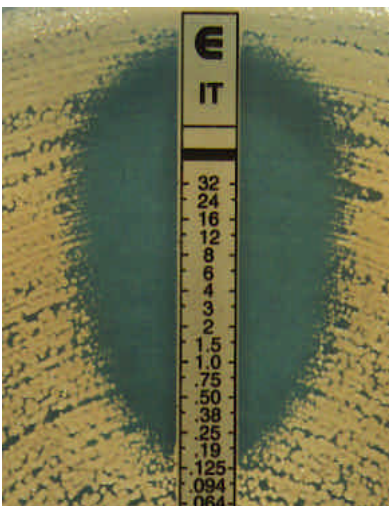


Figure 6a. MIC 0.125 µg/ml (sharp end point).

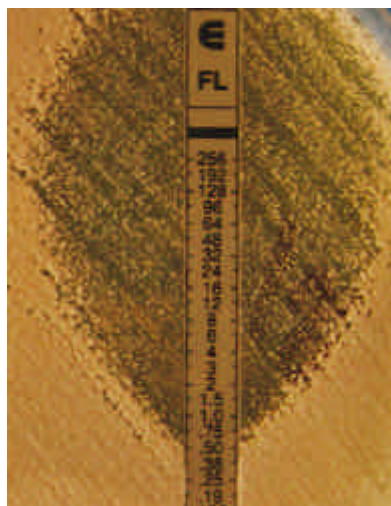


Figure 6b. MIC 0.5 µg/ml (lawn of microcolonies within a discernable ellipse).



Figure 6c. MIC >256 µg/ml (macrocolonies in ellipse).

QUALITY CONTROL

In order to check the performance of Etest with respect to the quality and consistency of reagents, media, inoculum and procedure used, test the quality control strains specified in table 1 as described under **Procedure**. Performance is satisfactory if MIC values obtained are within the quality control specifications provided.

Table 1. Etest quality control specifications for antifungal susceptibility testing.

Agent	Code	<i>C. krusei</i> ATCC 6258	<i>C. parapsilosis</i> ATCC 22019	<i>C. albicans</i> ATCC 90028 ¹⁾
Amphotericin B	AP	0.5-2	0.25-2	0.25-2
Flucytosine	FC	≥32	0.125-0.5	0.25-2
Fluconazole	FL	≥256 ²⁾	2-16 ³⁾	0.125-1
Itraconazole	IT	0.125-0.5	0.064-0.25	0.064-0.25
Ketoconazole	KE	0.25-1	0.032-0.125	0.008-0.064

Notes:

- 1) Use this strain to practise the selection of 80% inhibition with azole trailing end points.
- 2) An ellipse may be seen at 64 to 128 µg/ml with macro-colonies up to ≥256 µg/ml.
- 3) Occasionally, isolated colonies may grow up to 16 µg/ml.

Patient results should not be reported if quality control values are outside the stated ranges. Frequency of quality control testing should be established by the individual laboratory and further guidelines are provided by NCCLS documents M7, M11 and M100 series.

Perform regular colony counts to verify that your inoculum suspension contains the correct number of viable cells in cfu/ml. For example, dilute the appropriate inoculum suspension 1:1000 and subculture 1 µl onto Sabouraud Dextrose agar for colony counts. An acceptable inoculum would give approximately 100 to 500 colonies, i.e. 1 to 5x10⁸ cfu/ml.

Remember that Mc Farland turbidity standards do not guarantee correct colony counts of viable cells.

EXPECTED VALUES

Antifungal susceptibility levels for different biological populations are no longer predictable due to resistance development. Thus, the laboratory can only use the expected MIC values of the different antifungal agents for the quality control strains specified to ensure that clinical results obtained are reasonably accurate.

PERFORMANCE CHARACTERISTICS

Etest performance characteristics have been established with comparative evaluations at external clinical sites and *in house* testing. These studies have shown that Etest MIC values correlate with the NCCLS reference broth microdilution method. Etest is considered to be in essential agreement (EA) with the reference NCCLS procedure when MIC values from both methods show an EA of at least 90% within 2 dilutions.

LIMITATIONS

1. As with all methods of antifungal susceptibility testing, Etest results are *in vitro* values and can only provide an indication of the organism's potential *in vivo* susceptibility. The use of results to guide antimicrobial therapy selection must be the sole decision and responsibility of the attending physician who should base his or her judgement on the particular history and knowledge of the patient, the pharmacokinetics and pharmacodynamics of the antifungal agent and prior clinical experience in treating infections caused by the particular species of yeast and the agent being considered.
2. For details of specific interpretive limitations on susceptibility categories, the user is referred to information in the latest NCCLS Antifungal Susceptibility Testing document (M27-A, June 1997, Appendix C, p. 16).

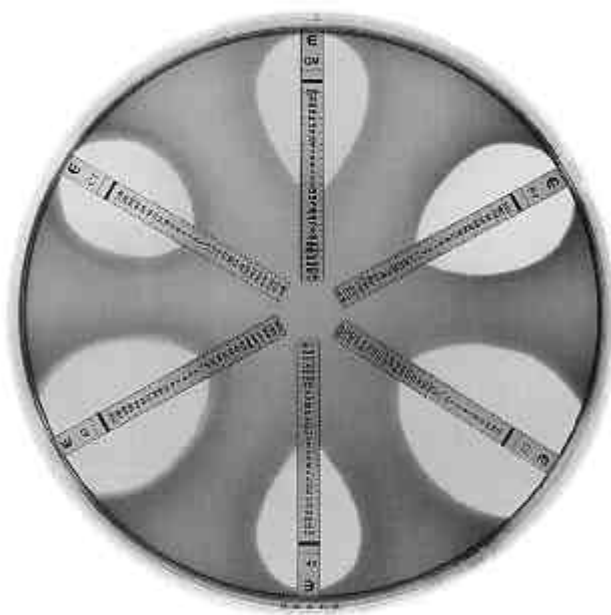
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Etest[®]



Etest for MIC determination of Antifungal Agents

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in all major markets.

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