Standard Operating Procedures

Mycology Laboratories

Antimicrobial Resistance Surveillance
and Research Network

This document contains detail procedures on sample collection, transport, isolation, identification of fungi for the diagnosis of invasive fungal infections and antifungal susceptibility testing of yeast

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CONTENTS

Sample Collection, Transport and Processing of Specimens

The principal goals of a sound clinical mycology laboratory are to isolate efficiently and to identify accurately the suspected etiological agents of fungal infection. Success depends much on the quality of clinical specimens sent to the laboratory.

The following points need to be emphasized: -

a) Appropriate sample/specimen collection

b) Prompt transportation

c) Correct processing of the specimen

d) Inoculation of specimens onto appropriate culture media and incubation at suitable temperature

Specimens should be collected aseptically, placed in sterile containers, delivered to the laboratory within 2 hours, processed, and then inoculated to primary isolation media within a few hours of collection. Viability may decrease with prolonged specimen storage.

Swabs are not encouraged; however, specimens from the environment or certain body sites such as the ear canal, nasopharynx, throat, vagina and cervix are not readily collected by other means. Swabs for collection of material from open wounds or draining lesions are frequently contaminated with environmental microorganisms.

All specimens sent to mycology laboratory must be clearly labeled with the patients' name, age, sex, unit number, date and time of collection, source of specimen, antimicrobial therapy together with a brief relevant clinical history and the name of the attending physician.

Collection of specimens for diagnosis of fungal infections

Specimen		**Collection**	**Unacceptable specimen**
a)	Pus	-Aseptically with needle and syringe from undrained abscess -Pus expressed from abscess opened with scalpel; transported to laboratory either in sterile container/ syringe and needle	Swab or materials from open wound
b)	Biopsy	-Place between two sterile gauze pads, sterile petri dish/ tube (containing 2-3 ml of sterile normal saline/ brain heart infusion broth) -Tissue is collected from centre and edge of the lesion	Swabs, sample collected in thioglycolate broth or formol saline
c)	Grains	-Collected by lifting the crust at the opening of a sinus. -Grains frequently found underneath the pus or collected from the removed bandages -Aspirated from undrained sinuses	
d)	Cerebrospinal fluid	-~ 3 ml in a sterile tube	Insufficient quantity
e)	Body fluids	-Sterile tube or in a heparinized syringe	Swabs

f)	Bone marrow	-~ 0.2-0.3 ml collected in a sterile heparinized syringe -Sterile cap is placed on heparinized syringe and transported immediately	Clotted bone marrow
g)	Blood	-~ 5-10 ml in yellow Vacutainer/syringe or in biphasic media containing brain heart infusion broth and agar; blood: broth ratio should be maintained at 1:10 -Multiple blood cultures at timed intervals to be collected - BACTEC/lysis centrifugation technique may improve sensitivity	
h)	Urine	-Early morning 25-50 ml of clean catch midstream urine specimen -Suprapubic aspirate, catheterized specimen -Collected in sterile container	24 hours collection is unacceptable
i)	Faeces	-Not usually acceptable in the Mycology laboratory. -Sometime collected to access *Candida* carriage in G I tract.	
j)	Sputum	-5-10 ml; early morning prior to eating -Use mouth rinse and brush before collection -Collected in sterile wide mouthed container.	Saliva, nasal secretion, throat swab, 24 hour collection
k)	Bronchial brush/washing/ broncho-alveolar lavage	-Collected in sterile container using fiber optic bronchoscopes	Dried specimen
l)	Lung biopsy	-Collected by bronchoscope, fluoroscope guided trans-thoracic needle aspiration or open lung biopsy -Best specimen is open lung biopsy but it is hazardous	
m)	Serology	-Serum – 1-2 ml with 1:100 parts merthiolate -3-5 ml of spinal fluid with same concentration of merthiolate	Specimen collected after skin test with histoplasmin while performing serology for histoplasmosis

Transport of specimens

1. Specimens should be transported in sterile, humidified, leak-proof container. Dermatological specimens, however, should be transported in a dry container. Transport medium should not be used unless the specimen can be easily and completely retrieved from the medium. Although fungi can be recovered at times from specimens submitted in anaerobic transport media, such media should be avoided.
2. Specimens should be processed and inoculated to primary isolation media as soon as possible after collection, ideally within few hours. It should not be presumed that successful methods

for storage of fungal cultures are suitable for temporary storage of clinical specimens that harbor relatively few fungal cells.

3. The effect of refrigeration on fungal specimens has not been well studied, but if processing is to be delayed for more than several hours, it is recommended that specimens be stored under refrigeration at 4°C with the following exceptions: blood and cerebrospinal fluid are stored at 30-37°C; dermatological specimens are stored at 15-30°C.

Processing of specimen in the laboratory

- Specimens should be first examined carefully. The examination allows for selection of the proper portion of the specimen that will likely contain the fungus. Caseous, purulent or bloody areas, and necrotic materials are processed.
- Specimens from cases of mycetoma are examined with the dissecting microscope for the presence of granules before processing (Fig. 1).
- Punch biopsies should be examined carefully to ensure that they are divided vertically and not horizontally so that each layer of tissue is represented in each specimen.
- Clinical specimens must be processed as soon and as carefully as possible on the appropriate isolation media and temperature. Recovery of pathogens is necessary for their identification and evaluation against the antifungal agents.
- Most specimens suspected of having fungi other than dermatophytes should be handled according to practices outlined for Biosafety Level 2.

Direct inoculation:

- Many specimens (up to 0.5 ml) can be directly inoculated to media
- Specimens like – abscess aspirate, bone marrow aspirates, cerebrospinal fluid, swabs, body fluid, hair, skin scraping, nail, bronchial washing or brushing etc. can be directly inoculated
- If the fluid has a clot or membranous material, mince with sterile scalpel and inoculate to media.
- 3-5 drops of fluid should be inoculated to each tube of media

Concentration:

- Large volume of fluids should be concentrated by centrifugation (1500-2000 X g for 5 minutes) before inoculation to isolation media as a means to enhance the detection and recovery of fungi.

Sputum:

Method: N-acetyl L- cysteine (NALC) treatment

Principle: NALC is mucolytic agent due to ability to split the disulfide bonds in the mucoprotein

Stock solution to be prepared:

1. 2.94% of Na-citrate in distilled water – autoclaved at 121°C x 15 minutes
2. 0.5 g of NALC in 100 ml of Na-citrate (to be freshly prepared)
3. M/15 phosphate buffer (pH 6.8-7.1)

Procedure: -

Specimen to be vortexed with sterile glass beads

↓

Equal volume of Na-citrate and NALC to be added to the specimen; to be vortexed again for 10-30 seconds

↓

Dilute mixture in the phosphate buffer by adding double the volume and centrifuge at 1000 g for 15 minutes

↓

Use sediment to prepare smears for direct microscopy and to inoculate on media for culture

Blood

Biphasic Medium

- At least 3-5 ml blood is cultured in biphasic medium (a ratio of 1:10 to 1:20 blood to broth is utilized)
- Two bottles can be utilized. One can be incubated at 25°C and the other at 37°C.
- After 48 hours, 5 days, and 7 days incubation, tilt the bottle in such fashion that fluid covers the whole agar surface; but does not reach the neck of the bottle. Keep the bottle in this position for one hour

Automated, continuously monitoring blood culture system

ESP system (Trek Diagnostics, Westlake, Ohio, USA), Bac T/Alert system (Organon Teknika Corp, Durham, NC, USA) and Bactec system (Becton Dickinson Microbiology system, Sparks, MD, USA)

5 ml of blood is sufficient

The Bactec Myco/F Lytic medium can be used both for Mycobacterium and fungi

Body fluid, urine:

Filtration or centrifugation at 2000 X g for 10 minutes

Exudates, pus, drainage:

Washing, centrifugation and crushing of granules

Cerebrospinal fluid

-Always culture 10-30 ml of CSF

-Centrifuge CSF - (culture & microscopy of sediment; supernatant may be used for antigen detection)

-Repeat lumbar puncture at least 3 times

-May collect sample even after start of therapy as fungi can be isolated till 3rd day of therapy

-Consider cisternal puncture if lumbar puncture is negative

Catheter tip for isolation of fungi:

- External dressing carefully removed
- Alcohol used to remove blood, or antibiotic ointment from the site of catheter insertion Catheter is withdrawn through the skin
- Sterile forceps may be used to remove the catheter
- Short intra-vascular catheters: Shaft cut off from the catheter hub with sterile scissor and dropped into a sterile vessel or cup (the catheter segment length should be 2.5 - 5 cm)
- Larger indwelling catheters: Distal 5 cm should be aseptically collected
- Purulent exudates: If present at the catheter exit site, a sample should be collected for Gram stain and culture
- Catheter tip rolled across the surface of the culture plate at least four times The laboratory shall report the colony count for the catheter culture
- Sub-cultures of *Candida* from line tips stored at 4°C and sent to central lab

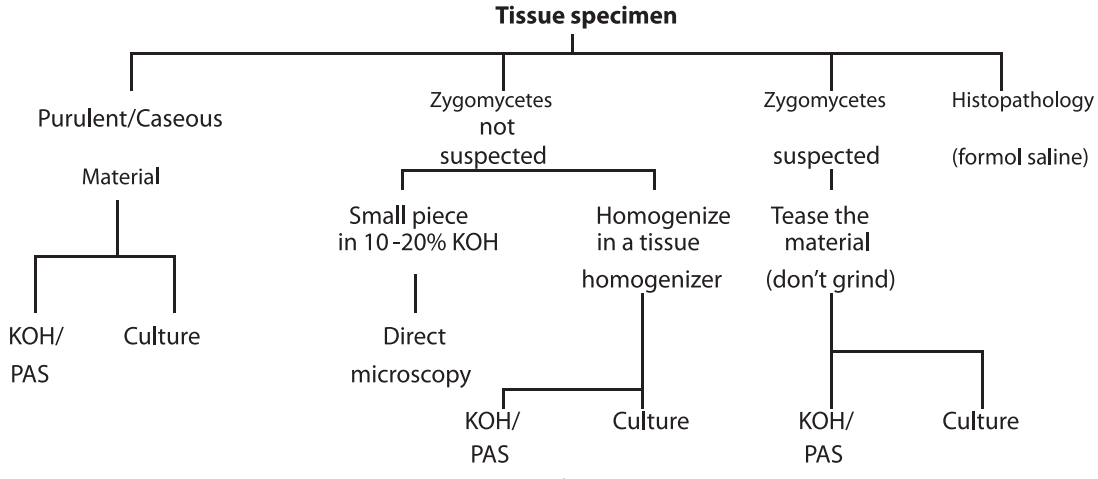

Fig. 1. Processing of Tissue Specimens

Direct microscopic examination and staining of clinical specimens

Direct microscopy is of immense importance for mycological investigation of clinical specimens. Unlike bacteria, fungi may take pretty long time to grow in culture. Moreover, many fungi are saprophytic. Therefore, clinical situation should be correlated with direct microscopy findings and culture.

Potassium hydroxide mounting fluid preparation:

The KOH preparation is probably the most important microscopic preparation used by the mycologist; it can also be the most difficult if expert does not interpret the findings. Advantages include: 1) speed with which it is made and examined, 2) reagents are easily obtainable; stable.

Preparation:

1. 10% Potassium hydroxide solution
    i. Potassium hydroxide 10 g
    ii. Glycerol 20 ml
    iii. Distilled water 80 ml

Quality control: Check the reagent. If cloudy, prepare a new solution. Using a portion of sputum specimen, ensure that the clinical material dissolves.

Procedure:

a) Place the material to be examined onto a clean glass slide.

b) Add a drop of 10% KOH to the material and mix

c) Place a cover slip over the preparation

d) Allow the KOH preparation to sit at room temperature until the material has been cleared. The slide may be warmed to speed the clearing process. Slides that are initially negative for fungi may be re-examined the following day.

e) Observe the preparation by bright field or phase contrast microscopy

KOH-Calcofluor white (CFW) solution mixture:

Calcofluor white preparation

Stock solution:

Calcofluor white	1 g
Distilled Water	100 ml

Working solution

1:10 dilution of stock solution in 0.05% Evans blue

Always store the stock and working solution in the refrigerator at 4°C.

Procedure:

a) Place the material to be examined onto a clean glass slide

b) Add a drop of KOH (10%) - Calcofluor solution (0.01%), or mix in equal volumes before processing.

c) Mix and place a cover glass over the material

d) If necessary, allow the KOH preparation to sit at room temperature (25°C) for a few minutes until the material has been cleared. The slide may be warmed to speed the clearing process.

e) Observe the preparation by UV microscopy. Calcofluor may not stain strongly dematiaceous fungi. If such is suspected, the preparation should be examined by bright field microscope. *Candida glabrata* may fluoresce only very faintly. Elastin and collagen will also fluoresce, but with a yellow-green fluorescence.

Quality control:

1. Check the reagent prior to use, weekly, and with each new batch of calcofluor prepared.
2. Using an aqueous suspension of actively growing *Candida albicans*, the yeast cell walls will be bright green or blue white depending upon ultraviolet filters used.
3. Negative control consists of KOH and calcofluor combined.

India Ink/Nigrosin:

India ink is obtained commercially, usually from any store that handles art supplies. The Mycology Training Branch has found Pelikan Drawing Ink to be excellent. The India ink (Sanford/ Pelikan) may be preserved with 0.5% phenol.

Nigrosin (granular)	10.0 gm
Formalin (10%)	100 ml

- Place the solution in a boiling water bath for 30 minutes. Add 10% formalin lost by evaporation
- Filter twice through double filter paper (Whatman No. 1)

Procedure: Equal parts of the patients' sample (CSF, other body fluids, urine etc.) mixed with India ink/nigrosin on a clean glass slide. The preparation should be brownish color, not black. If the preparation is too black, 2-3 loop full of sterile distilled water to be mixed.

A control smear should always be made with India ink only without adding any sample.

Interpretation: Presence of budding yeast cells with a clear halo around them is indicative of capsular material and is considered a positive India ink preparation. When such yeasts are seen in spinal fluid sediment, it is suggestive of *C. neoformans* although cultures are needed to confirm the identity of these yeasts.

White cells may also repel carbon particles but the halo has a fuzzy, irregular appearance at the periphery.

Gram stain:

Gram stain is usually a poor stain to use when examining a specimen for a fungus. Gram stain may be used when examining smears of *Candida, Malassezia*, and *Sporothrix* but should not be relied upon to demonstrate the yeasts of the other dimorphic fungi. All fungi are gram positive. It is most useful for demonstrating mycelial elements and budding yeasts cells in sputa, vaginal secretions, purulent material, gastric washing, lung aspirates and urine.

Gram stain reagents

1. Crystal Violet Reagents

Crystal violet (85% dye)	2 gm
Ethyl alcohol (95%)	10 ml
Distilled water	100 ml

2. Grams Iodine Solution

Iodine	1 gm
Potassium iodide	2 gm
Distilled Water	300 ml

3. Counter Stain

Safranin O	1 gm
Ethyl alcohol (95%)	40 ml
Distilled Water	400 ml

4. Decolorizer

Acetone or Alcohol

Procedure

- Heat fix the material on the slide
- Flood the slide with crystal violet for 30 seconds to 1 minute. Rinse with tap water.
- Flood the slide with iodine for 30 seconds. Rinse with tap water.
- Decolorize with alcohol or acetone and rinse off immediately with tap water.
- Counter stain with safranin for 30 seconds. Rinse with tap water and allow it to dry.
- Examine under oil immersion.

Giemsa Stain:

This stain is used when intracellular structures are to be examined like the yeast cells of *Histoplasma capsulatum*. The intracellular cells of *H. capsulatum* stain light to dark blue and have a hyaline halo. The halo is not a capsule, rather a staining artifact. This stain can also be used to visualize trophozoite stage of *Pneumocystis carinii* (*jiroveci*). It is a compound stain formed by the interaction of methylene blue & eosin.

Preparation

Giemsa powder	600 mg
Methyl alcohol (Acetone free)	50 ml
Glycerine	50 ml

- Grind Giemsa powder in mortar. Pour methyl alcohol and glycerine and decant from top.
- Grind it again with glycerine till whole stain is dissolved.
- Keep the stain at 55°C for 2 hours; shaking gently at 30 minutes interval.
- Keep it for two weeks for maturation.

Buffer Solution

Na_2HPO_4	6.77 gm
KH_2PO_4	2.5 gm
Distilled Water	1000 ml
pH	7.2

Procedure

- Dilute the stain with buffer for use.
- Fix smear with absolute methyl alcohol.
- Put stain on smear for 15 minutes.
- Blot dry and examine.

Interpretation: Fluids, exudates or buffy coats should be spread evenly over a slide and impression smear may be prepared from tissues.

Periodic acid Schiff (PAS) stain:

It is histopathological stain used to detect fungi in clinical specimens, especially yeast cells and hyphae in tissues. The staining of fungi is based on Feulgen reaction. Periodic acid or chromic acid of PAS stain oxidizes the aldehyde groups to form polyaldehydes; which yield purple red colour on contact with Schiff's fuchsin sulphite.

Formulation

(a) Periodic acid solution

Periodic acid	5.0 gm
Distilled water	100 ml

(b) Basic fuchsin Solution

Basic fuchsin	0.1 gm
Ethanol (absolute)	5.0 gm
Distilled water	95.0 ml

* Mix ethanol and distilled water in a brown bottle; add basic fuchsin and mix by rotating.

(c) Sodium Metabisulphite Solution

- Sodium metabisulphite 1.0 gm
- Hydrochloric acid 10.0 ml
- Distilled water 190 ml

Add HCl to distilled water in a brown bottle

- Add sodium metabisulphite

Procedure

Slide (fix film by flaming).

Immerse in ethanol for 1 minute.

Place in 5% periodic acid for 5 minutes.

Wash gently in running tap water for 2 minutes.

Pour basic fuchsin for 2 minutes.

Wash in running tap water for 2 minutes.

Pour sodium metabisulphite for 3-5 minutes.

Wash in running tap water for 5 minutes.

(counterstain with dilute aqueous malachite green if desired).

Dehydrate in 70%, 80%, 95%, 100% ethanol.

Xylene: 2 minutes.

Mount with permount.

Controls for stain: A slide of either skin or nail scrapings containing a dermatophyte should be stained along with slides of the specimen. Periodic acid may deteriorate and no longer oxidizes the hydroxyl groups. This should be suspected when fungal elements on the control slide appear unstained. The Periodic acid solution should be kept in a dark bottle. The stock of periodic acid (a white powder) should be stored in a desiccator. The sodium metabisulphite solution is unstable. Deterioration of this reagent is suspected when the control slides show no evidence of having been subjected to a bleaching process e.g. background stains as intensely as does fungal elements. The basic fuchsin solution is stable and rarely the source of any problem in this stain.

Methenamine silver stain:

Gomori's methenamine silver stain is perhaps the most useful stain for visualizing fungi in tissue.

Material required

4% Chromic acid

1% Sodium / Potassium metabisulphate

0.1 Ferric chloride ($FeCl_3$)

5% Sodium thiosulphate

1% Light green

Stock solution of hexamine*;*

3% Hexamine in 5% silver nitrate ($AgNO_3$).

Working solution of hexamine

Stock 25 ml

5% Borax – 2 ml

Distilled Water- 2 ml

Procedure

Dry the smear.

Fix in methanol for 5 minutes.

Wash in distilled water.

Add 4% chromic acid for 45 minutes. Wash in distilled water.

Add 1% sod/pot metabisulphite for 1-2 minutes (or till smear becomes colourless). Wash in distilled water.

Keep in working solution of hexamine (which is preheated at 56°C) for one hour (smear becomes dark brown).

Wash with distilled water till smear becomes black then wash with 0.1% $FeCl_3$.

Wash with 5% sodium thiosulphate for 2 minutes.

Wash with distilled water.

Pour 1% Light green for 1 minute.

Wash with distilled water.

Dry and see under oil immersion.

Result: Fungal element stains black; background stained – green.

Yeast Identification

Yeasts are heterogeneous fungi that superficially appear as homogenous. Yeasts grow as unicellular form and divide by budding, fission or a combination of both. The various yeasts are distinguished from each other based upon a combination of morphological and biochemical criteria. Morphology and the methods of asexual reproduction are primarily used to identify genera, whereas biochemical tests are used to differentiate the various species.

Approaches for the identification of the yeasts includes:

1. Culture characteristics- Colony color, shape and texture.
2. Asexual structures -
    - Shape and size of cells
    - Type of budding - unipolar (*Malassezia)*, bipolar *(Hansaniaspora kloekera*) multipolar (*Candida*) , fission (*Schizosaccharomyces*)
    - Presence or absence of arthroconidia, blastoconidia, ballistoconidia, clamp connections, germ tubes, hyphae, pseudohyphae, sporangia or sporangiospores.
3. Sexual structures- Arrangement, cell wall, ornamentation, number, shape and size of ascospores or basidiospores.
4. Physiological studies-
    - Sugar fermentation
    - Sugar assimilation
    - Nitrogen utilization
    - Urea hydrolysis
    - Temperature studies
    - Gelatin liquefaction

Conventional Identification Procedures:

1. Isolation techniques for mixed cultures

Before proceeding for the identification of the yeast it is necessary to purify the yeast because initially isolated yeast may be contaminated or in mixed culture.

2. Direct mounts

Direct mounts are made to study yeast microscopic morphology and to determine the purity of the isolates. Direct mounts are made using Lactophenol Cotton Blue (LCB).

3. Germ-tube test

Germ-tube test is a simple, reliable and economical procedure for the presumptive identification of *Candida albicans.*

Procedure:

- Take 0.5 – 1 ml of serum (pooled human, sheep, fetal calf, bovine, horse or egg albumin) into a 12×75 mm test tube.
- Suspend the yeast colonies into the serum to obtain faintly turbid suspension.

- Incubate the tubes at 37 °C for 2-3 hours in a water bath/ incubator.
- Using sterile Pasteur pipette, remove the suspension and examine it microscopically for the presence or absence of germ tubes.
- *Candida albicans* and *C. tropicalis* are inoculated with each group of germ tube determination to serve as positive and negative controls respectively.
- Germ tubes arise directly from the yeast cell and have parallel walls without any constriction at their point of origin.

4. Morphological characters on Corn Meal Agar (Dalmau plate)

- Prepare Corn meal agar containing 1% Tween 80 in a 90 mm plate. Divide the plate into 4 quadrants and label each quadrant.
- Using a sterile needle or straight wire, lightly touch the yeast colony and then make 2-3 streaks of approximately 3.5 – 4 cm long and 1.2 cm apart.
- Place a flame sterilized and cooled 22 mm square cover glass over the control part of the streak. This will provide partially anaerobic environment at the margins of the cover slip.
- Incubate the plates at 25 °C for 3-5 days.
- Remove the lid of the petri plate and place the plate in the microscope stage and observe the edge of the cover glass using low power objective (10X) first and then high power objective (40X) .
- Morphological features like hyphae, pseudohyphae, blastospores, ascospores, chlamydospores, basidiospores or sporangia are noted.

5. Ascospore Production & Detection test:

Identification of yeast also involves determining whether or not the isolate has the ability to form ascospores. Some yeast will readily form ascospores on primary isolation medium whereas others require special media. Ascospores are produced under limited nutrients in the media.

The commonly used media are:

- Malt extract agar (5% malt extract and 2% agar)
- Acetate agar (0.5% sodium acetate trihydrate and 2% agar pH 6.5 - 7.0)
- V-8 juice agar (commercially available)

Procedure: -

- Inoculate the yeast onto ascospores producing agar plates.
- Incubate aerobically at 25°C.
- Examine the culture in 3-5 days and weekly thereafter for 3 weeks.
- Prepare wet mount of the yeast in distilled water.
- Examine the wet mount under oil-immersion objective.
- Observe for ascospores form, surface topography, size, color, brims and number of ascospores per ascus.
- If the ascospores are not seen in a wet mount, perform modified acid-fast stain (ascospores are acid fast).

- *Saccharomyces cerevisiae* should be included as positive control for production on media and staining procedure.

6. Sugar Fermentation: At least 6 sugars (Table -1) should be used for the fermentation test Procedure:

- Prepare liquid fermentation medium containing peptone (1%), sodium chloride (0.5%), Andrade's indicator (0.005%). Sterilize by autoclaving at 120 C for 15 minutes at 15 pounds pressure. Add filter-sterilized sugar at the concentration of 2% to the medium. Pour into the sterile test tubes (approx. 5 ml) and place sterile Durham's tube into each tube.
- Plug the tubes with colour coded cotton plugs.
- Inoculum preparation is done by suspending heavy inoculum of yeast grown on sugar free medium.
- Inoculate each carbohydrate broth with approximately 0.1 ml of inoculum.
- Incubate the tubes at 25°C up to 1 week. Examine the tubes every 48-72 hours interval for the production of acid (pink color) and gas (in Durham's). Production of gas in the tube is taken as fermentation positive while only acid production may simply indicate that carbohydrate is assimilated.

7. Sugar assimilation test (Auxanographic technique)

At least 12 sugars (Table -1) should be tested for assimilation

- Preparation of yeast nitrogen base

Yeast nitrogen base is prepared using the ingredients as follows:-

Potassium dihydrogen orthophosphage (KH_2PO_4)	-	1.0 gm
Magnesium sulfate ($MgSO_4$)	-	0.5 gm
Ammonium sulphate (NH_4SO_4)	-	5.0 gm
Noble agar	-	25.0 gm

Autoclave at 115 °C for 15 minutes.

OR

- IF YNB is obtained from Difco, prepare YNB and agar separately as follows:

1. YNB (Difco) - 6.7 gm

   Distilled water - 100 ml

2. Agar - 20.0 gm

   Distilleed water - 980 ml

Dispense in 18 ml quantities in 18 × 150 mm screw-capped tubes. Autoclave 121 °C and store at 4 °C.

- Prepare a yeast suspension from a 24-48 hours old culture in 2 ml of YNB by adding

heavy inoculum. Add this suspension to the 18 ml of molten agar (cooled to 45°C) and mix well. Pour the entire volume into a 90 mm petri plate.

- Allow the petri plate to set at room temperature until the agar surface hardens.

- Place the various carbohydrate-impregnated discs onto the surface of the agar plate.
- Sugar discs can be obtained commercially or can be prepared as follows:

Punch 6 mm-diameter disc from Whatman no. 1 filter paper. Sterilize the disc by placing them in hot air oven for 1 hour. Add a drop of 10% filter sterilize sugar solution to each disc. Dry the disc at 37°C and store at 4°C in airtight container.

- Incubate the plates at 37°C for 3-4 days.
- The presence of growth around the disc is considered as positive for that particular carbohydrate. Growth around glucose disc is recorded first which serves as positive control (viability of yeast).

8. Nitrate Assimilation Test (Auxanographic technique): -

Preparation of Yeast Carbon base:

Potassium dihydrogen orthophosphate (KH_2PO_4)	-	1 gm
Magnesium sulfate ($MgSO_4$)	-	0.5 gm
Glucose	-	20 gm
Noble agar	-	25 gm
Distilled water	-	1 L

Mix the reagents by boiling. Autoclave at 121°C for 15 minutes.

OR

Prepare yeast carbon base and agar separately:

a).	Yeast carbon base	-	11.7 gm
	Distilled water	-	100 ml

Sterilize by filtration and store at 4°C until use.

b) .	Agar	-	20 gm
	Distilled water	-	980 ml

Autoclave at 121°C for 15 minutes. Dispense in 18ml quantities in 18 X 150 mm screw-capped tubes.

- Make a yeast suspension in molten YCB agar and pour into 90 mm diameter petri plate and allow to cool.

OR

- Prepare the suspension from a 24-48 hours culture in 2 ml of YCB equal to Mc Farland No. 1 standard. Add this suspension to 18 ml of molten agar cooled at 45oC. Mix well and pour into 90 mm petri plates.
- Allow the plate to cool to room temperature.
- Divide the plate into two halves and label them.
- Place the potassium nitrate disc (KNO_3) and peptone disc on the surface of the agar on corresponding labeled halves.

- Potassium nitrate discs are prepared as follows:

  Potassium nitrate - 30 gm

  Distilled water - 1 L

Mix the reagents and autoclave at 15 psi for 15 minutes. Take 6 mm disc (punched from Whatman No. 1 filter paper) and saturate with the potassium nitrate solution and dry the discs in sterile petri plate and store at 4°C until use.

- Peptone disc is prepared similarly as KNO_3 disc.

  Peptone - 30 gm

  Distilled water - 1 L

Incubate the plate at 37°C for 7 days. Check for the growth around the disc. The test is considered valid if the growth is present around the peptone. The growth around the KNO3 impregnated disc is considered positive.

9. Urea Hydrolysis:

- The Christensen's urea agar is recommended and is prepared according to the manufacturer instructions. Using a loop inoculate a small amount of the yeast colony on the agar surface.
- Inoculate appropriate control (*C. albicans* - negative control, *C. neoformans* - positive control).
- Incubate the slant at 25°C for 2-5 days. Read the test and the control tubes.
- A deep pink color indicates a positive test.

10. Rapid Urea Hydrolysis:

The rapid urea hydrolysis test is done in microtitre plates and used to screen for *C. neoformans.*

- Reconstitute each vial of Difco urea® broth with 3 ml of sterile water on the day to be used.
- Dispense 3-4 drops into each well of the microtitre plate.
- Transfer a heavy inoculum of freshly isolated colony of yeast into urea broth into a well.
- Seal the plate with a tape and incubate for 4 hours at 37°C.
- Pink to red color is positive test.
- Similarly inoculate the controls, *C. neoformans* as positive control and *C. albicans* or uninoculated well as negative control.

11. Temperature Studies:

Another important step in the identification of the yeast is by determining the ability to grow at an elevated temperature. It can be used to distinguish *C. neoformans* from other species of *Cryptococcus* and *C. dubliniensis* from *C. albicans*.

Procedure: -

- Inoculate two tubes of malt extract agar with the isolate.
- Incubate one tube at 37°C and other at 25°C.
- Examine the tube everyday up to 4-7 days for the presence of growth.

- Growth must be present in both the tubes before concluding that the yeast has the ability to grow at 37°C.

Yeast identification by Vitek 2 automated system

Procedure:

Inoculum preparation:

- Use 24 – 48 hours old pure culture of the yeast grown on Blood agar or Sabouraud's dextrose agar
- Dispense 3 ml of suspension solution (provided by manufacturer) into the disposable plastic tubes provided by the manufacturer
- Emulsify one or two colonies of the yeast in the suspension solution using a sterile inoculation loop
- Check the turbidity of the suspension with the help of the densitometer (Densichek). The optical density of the suspension should be in the range of 1.8 to 2.2 Mc Farland. Repeat inoculum preparation if the optical density is out of range.

Loading the inoculum:

- Arrange the inoculum tube (s) in the cassette
- Place a new Yeast ID card in the suspension through the capillary. The capillary should be immersed into the suspension
- Load the cassette tray into the loading chamber of the Vitek 2 machine
- Close the loading chamber door and press 'start fill' button. The suspension will be absorbed into the card through the capillary by vacuum inside the chamber
- After the beep, transfer the card from the loading chamber to the incubator chamber Close the door and give accession number (s) and sample details of the isolates in the computer. Press 'save data' option.
- Remove the cassette after cards are transferred to the incubator and discard the tubes in the biohazard disposal bag
- Read the identification results after 18 and half hours.
- Discard the used cards from the waste bucket chamber for disposal.

Interpretation of result:

- Check the results by opening the result tab of the Vitek 2 program in the computer
- Note down the species identity and the percentage identity in the register
- For low discriminatory and low percentage identity results, proceed for further confirmatory test using the molecular methods as described below.

Molecular identification of yeasts

STEP 1: Isolation of genomic DNA from yeast

- Subculture a pure culture of yeast on SDA and incubate for 48 hours, scrape off the growth by an inoculation loop and suspend a loopful in 400 µL breaking buffer.

- Add approximately 0.3 g of sterile glass beads and vortex the mixture vigorously for 5-10 minutes.
- Add 1 µL RNAse (10 mg/mL) and incubate at 37°C for 1 hour in waterbath. Add 1 µL of Proteinase K (20.2 mg/mL) and incubate at 55°C for 1 hour.
- Add approximately equal volume of Phenol: chloroform mixture (1:1) and centrifuge at 12000 rpm for 10 minutes at room temperature.
- Take out upper aqueous phase with a sterile pipette into a sterile microcentrifuge tube and add equal volume of chloroform: Isoamyl alcohol mixture (24:1) to it.
- Centrifuge the mixture at 12000 rpm for 10 minutes.
- Collect the aqueous phase and add 1/10th volume of 3 M sodium acetate solution and equal volume of chilled isopropanol, mix it and incubate at -20°C for 2 hours or keep overnight at 4°C to precipitate the DNA.
- Collect the precipitated DNA by centrifuging at 13000 rpm for 5 -10 minutes at 4°C and decant the supernatant
- Add 0.5 mL of 70% ethanol to the precipitate and centrifuge at 13000 rpm for 5 minutes to wash the pellet. Aspirate out the supernatant carefully and dry the pelleted DNA at 37°C for 1 hour.
- Re-suspend the pellet DNA in appropriate volume of Tris EDTA buffer (TE) , dissolve at room temperature with intermittent mixing.
- Finally, electrophorese the DNA on 0.8% agarose gel to check the quality and quantity of DNA, spectrophotometrically by Nano drop 2000

STEP 2: PCR amplification**.**

Amplify DNA of large ribosomal subunit LSU (rDNA) gene or internal transcribed spacer (ITS) in a thermal cycler (Eppendroff mastercycler gradient, Germany) using pan-fungal primers for rDNA, NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3) targeting D1/D2 region of 26S rDNA of large ribosomal subunit; and ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3) and ITS-4 (5′TCCTCCGCTTATTGATATGC-3′) for the internal transcribed spacer regions (ITS-1 and ITS-2) between the small (18S) and large subunit (26S) rRNA genes respectively.

	PCR Reaction Mixture (for reaction mixture of 100 µL)			
S.NO.	REAGENT	INITIAL CONC.	FINAL CONC	Volume used.
1	PCR Buffer	10X	1X	10 µL
2	Forward primer	10 picoMole	0.2	2 µL
3	Reverse primer	10 picoMole	0.2	2 µL
4	DNTP mix	10 milliMolar	0.2	2 µL
5	Taq polymerse	1000 units/mL	0.5 units	0.5 µL
6	MilliQ water			83.5 µL

Template used = 0.5-1.0 µL (according to the conc. of template)

- In case of NL-1 and NL-4 set of primers use following PCR program

Step Cycles

1. T=95°C for 5 minutes.

2. T=94°C for 1 minute.
3. T=55°C for 30 seconds.
4. T=72 °C for 2 minutes
5. GO TO STEP 2 REPEAT 35 CYCLES
6. T = 72 for 5 minutes
7. 6. 20°C HOLD

- In case of ITS-1 and ITS -4 set of primers use following cycle program

Step Cycles

1. T=95°C for 5 minutes.
2. T=94°C for 1 minute.
3. T=55°C for 30 seconds.
4. T=72 °C for 1 minutes
5. GO TO STEP 2 REPEAT 40 CYCLES
6. T = 72 for 7 minutes
7. 20°C HOLD

- Electrophorese the amplified products on 1% agarose gel and view the size and quality of PCR products in UV transilluminator or gel documentation system

STEP 3: Gel extraction of PCR Products

Excise the amplified products from the gel using QIAquick Gel Extraction Kit (Qiagen, Hilden) or other kits as per manufacturer instructions.

STEP 4: Sequencing PCR

Pre Requisite step:

Step I: DNA quantity

This is the initial step in which quantification of purified DNA (Qiagen purified kit), by measuring at 260 nm (Thermo Nanodrop 2000) or by 1.5% agarose gel electrophoresis, is done.

Step II: Template Quantity

The Table below shows the amount of template to use in a cycle sequencing reaction:

Template	**Quantity**
PCR Product:	
100-200 bp	1-3 ng
200-500 bp	3-10 ng
500-1000 bp	5-20 ng
Plasmid:	150-300 ng

Note:

- Higher DNA quantities give higher signal intensities.

- Too much template makes data appear too heavy, with strong peaks at the beginning of the run that fade rapidly
- Too little template or primer reduces the signal strength and peak height.
- The template quantities given above will work with all primers.

Steps in sequencing PCR:

- Start with purified DNA from step 3 as template for sequencing PCR. (Refer page32, step 3)
- Set up primers to a concentration of 6 pm/µl, dilute the primer in 1X TE Buffer
- Take a clean autoclaved 0.5 ml microcentrifuge tube

Set up reaction as follows:

Quantity	Reagents
X µl	H_20
1.75 µl	5X Buffer
Y µl	Template
1.0 µl	Big-Dye Terminator (3.1/ 1.1) (1:8 Dilution)
1 µl	Sequencing Primer (6 pm/ µl)
10 µl	Total

Note:

- Where quantity of X and Y depends upon the concentration of the template
- For 1. 300-600 bp size use 3.1 version BDT

  2. 100-250 bp size use 1.1 version BDT
- Gently mix and centrifuge so that mix gets sediment in the bottom
- Place the tube in a thermal cycler and set the volume to 10 µl and 0.5 ml tube selection
- Run on PCR machine using following conditions:

Step Cycles

1. T=96°C for 1 minutes.
2. T=96°C for 10 seconds.
3. T=50°C for 05 seconds.
4. T=60°C for 4 minutes.
5. GO TO STEP 2 REPEAT 25 CYCLES
6. 4°C HOLD

- Then start the run in the thermal cycler
- After the run is complete, centrifuge the tube briefly to bring the contents to the bottom of the wells.

- Store the tube according to when you are continuing the protocol:

Note:

- Within 12 hours – store at 0 to 4 °C
- After 12 hours – store at -20°C for 2 to 3 days
- After storage and before opening the tube, centrifuge the tube briefly to bring the contents to the bottom.

STEP 5: Purification of sequencing PCR Product:

Ethanol /EDTA/Sodium Acetate Precipitation

- Short spin the sequencing PCR product at 13000 rpm for 1 minute
- Add 2.0 µl 3M Sodium acetate (pH 4.6), 2.0 µl of 125 mM EDTA (Freshly prepared) and 10 µl MilliQ water in 10 µl of Sequencing PCR reaction.
- Mix well with tapping and briefly centrifuge
- Add 70 µl of absolute ethanol and mix properly by inverting the tube 10-35 times
- Important: Do not centrifuge at this step
- Incubate the tube for 15-20 minutes at -20°C
- Centrifuge at 13,000 rpm for 30 minutes
- Aspirate the supernatant immediately without disturbing the pellet
- Add 170 µl of 70% ethanol (freshly prepared) and wash the pellet by inverting the tube 50 times gently
- Centrifuge at 13,000 rpm for 15 minutes
- Aspirate the supernatant immediately without disturbing the pellet Repeat step 8 to 10 once.
- Dry the pellet at room temperature
- Important: There should be no ethanol left in this step.
- To continue, suspend the sample in 10 µl HiDi (Formamide provided by ABI) and mix the pellet properly and short spin and incubate the mix at 37°C for 15 minutes and again mix the pellet by tapping and quick spin the mix. Heat the reaction at 95°C to denature the amplicons for 5 minutes and quick chill in ice for 10 minutes.

Note: Sample can be stored in this step at 4°C

STEP 6: Capillary Electrophoresis

- Analyze the reaction products on ABI Prism 3130 automated DNA analyser (Applied Biosystems, California, USA) . Load the sample in 96 well plate according to the well description and make the entry of the sample in hard copy as well as in the sequencer data collecting software according to the well description

Use the DNA sequences so obtained for identification using nucleotide Blast search of NCBI (http://blast.ncbi.nlm.nih.gov/blast) or Centraalbureau voor Schimmelcultures (CBS) data base (http://www.cbs.knaw.nl/).

Table 1: Yeast Identification Chart

Species	Growth at 37OC	Pellicle in broth	Pseudo-/true hyphae	Chlamydospores	Germ tubes	Capsule, India ink	Assimilation of: Glucose	Maltose	Sucrose	Lactose	Galactose	Melibiose	Cellobiose	Inositol	Xylose	Raffinose	Trehalose	Dulcitol	Fermentation of: Glucose	Maltose	Sucrose	Lactose	Gala Galactose	Trehalose	Urease	KNO_3 Utilization	Phenol Oxidase	Ascospore
C.albicans	+	-	+	+	+	-	+	+	+	-	+	-	-	-	+	-	+	-	F	F	-	-	F	F	-	-	-	-
C. catenulata	+*	-	+	-	-	-	+	+	-	-	+	-	-	-	+	-	-	-	F*	-	-	-	-	-	-	-	-	-
C. dubliniensis	+	-	+	+	-	-	+	+	+	-	+	-	-	-	+*	-	+	-	F	F	-	-	F	F	-	-	-	-
C. famata	+	-	-	-	-	-	+	+	+	+*	+	+	+	-	+	+	+	+*	W	-	W	-	-	W	-	-	-	-
C. glabrata	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	F	-	F	-	F	F	-	-	-	-
C. guilliermondii	+	-	+	-	-	-	+	+	+	-	+	+	+	-	+*	+	+	+	F	-	F	-	F*	F	-	-	-	-
C. kefyr	+	-	+	-	-	-	+	+	+	+	+	-	+*	-	+	+	-	+	F	-	F	F*	F	-	+	-	-	-
C. krusei	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	F	-	-	-	-	-	-	-	-	-
C. lambica	+*	+	+	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	F	-	-	-	-	-	-	-	-	-
C. lipolytica	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. lusitaniae	+	+	+	-	-	-	+	+	+	-	+	-	+	-	+	-	+	-	F	-	F	-	F	F	-	-	-	-
C. parapsilosis	+	+	+	-	-	-	+	+	+	-	+	-	-	-	+	-	+	-	F	-	-	-	-	-	-	-	-	-
C. pintolopesii	+	+	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-
C. rugosa	+	+	+	-	-	-	+	+	-	-	+	-	-	-	+*	-	-	-	-	-	-	-	-	-	-	-	-	-
C. tropicalis	+	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	+	-	F	F	F	-	F*	F	-	-	-	-
C. zeylanoides	-	-	+	-	-	-	+	+	+	-	+	-	-*	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
C.neoformans	+	-	R	-	-	-	+	+	+	-	+	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
C.albidus	-	-	-	-	-	+	+	+	+*	+*	+	-	+	+	+	+*	+	+	-	-	-	-	-	-	+	+	+	-
C.laurentii	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
C.luteolus	-	-	-	-	-	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
C.tereus	-*	-	-	-	-	+	+	+*	+	+*	+*	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	-
C.uniguttulatus	-	-	-	-	-	+	+	+	-	-	+*	-	-*	+	+	+	+	+	-	-	-	-	-	-	+	+	+	-
R.glutinis	+	-	-	-	-	-*	+	+	+	-	+*	-	+*	-	+	+	+	+	-	-	-	-	-	-	+	+	+	-
R.rubra	+	-	-	-	-	-*	+	+	+	-	+	-	+*	-	+	+	+	+	-	-	-	-	-	-	+	+	+	-
S. cerevisiae	+	-	-*	-	-	-	+	+	+	-	+	-	+	-	+	+	+	+	F	F	F	F	F	F*	+	-	-	-
P.anomala	+*	-	-	-	-	-	+	+	+	-	+	-	+	-	+	+	+	+	F	F*	F	F	F	-	-	+	-	-
G. candidum	-*	+	+	-	-	-	+	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
B. capitatus	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P.wickerhamii	+	-	-	-	-	-	+	+	-	-	-	-	-	-	W	-	+	-	-	-	-	-	-	-	-	-	-	-
S.salmonicolor	+*	+	+*	-	-	-	+	+	+	-	+	-	+	-	+*	+	+*	+	-	-	-	-	-	-	+	+	-	-
T.asahi	+		+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-
T.mucoides	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	-	-	-
T.ovoides	+*	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	-	-	-

Antifungal Susceptibility Testing

Broth Dilution Antifungal Susceptibility Testing of Yeasts:

The method of antifungal susceptibility testing described here is intended for testing yeasts isolated from disseminated invasive infections. These yeasts comprise mainly *Candida* species, and *Cryptococcus neoformans*. This method has not been approved and used to test the yeast form of dimorphic fungi.

Broth microdilution method will be performed for amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, caspofungin, anidulafungin, micafungin following M27/A3 protocol of CLSI

Procedure of broth dilution method

1. Antifungal Agents

i. **Source:** Antifungal standards or reference powders can be obtained commercially, directly from the drug manufacturer or from reputed company as pure salt. Pharmacy stock or other clinical preparations / formulations **should not** be used. **Acceptable powders bear a label that states the drug's generic name, its assay potency [usually expressed in micrograms (µg) or International Units per mg of powder], and its expiration date** . The powders are to be stored as recommended by the manufacturers, or at -20° C.

ii. **Weighing Antifungal Powders:** All antifungal agents are assayed for standard units of activity. The assay units can differ widely from the actual weight of the powder and often differ within a drug production lot. Thus, a laboratory must standardize its antifungal solutions based on assays of the lots of antifungal powders that are being used. Either of the following formulae may be used to determine the amount of powder or diluents needed for a standard solution:

$$\text{Weight (mg)} = \frac{\text{Volume (mL)} \times \text{Concentration (µg/mL)}}{\text{Assay potency (µg/mg)}} \qquad \text{(formula 1)}$$

$$\text{Volume (mL)} = \frac{\text{Weight (mg)} \times \text{Assay potency (µg/mg)}}{\text{Concentration (µg/mL)}} \qquad \text{(formula 2)}$$

The antifungal powder should be weighed on an analytical balance that has been calibrated with Standards. Usually, it is advisable to accurately weigh a portion of the antifungal agent in excess of that required and to calculate the volume of diluents needed to obtain the concentration desired.

Example: To prepare 100 ml of a stock solution containing 1280 µg/mL of antifungal agent with antifungal powder that has a potency of 750 µg/mg, use the first formula to establish the weight of powder needed:

iii. Potency: it is advisable to weigh a portion of the powder in excess of that required, powder should be deposited on the balance until 182.6 mg was reached. With that amount of powder weighed, formula (2) above is used to determine the amount of diluent to be measured:

$$\text{Volume (mL)} = \frac{\text{(Powder Weight, 182.6 mg)} \quad X \text{ (Potency, 750 µg/mg)}}{\text{(Desired concentration, 280 µg/mL)}} = 107 \text{ ml}$$

Therefore, the 182.6 mg of the antifungal powder is to be dissolved in 107.0 ml of diluent.

iv. **Preparing Stock Solutions**: Antifungal stock solutions are prepared at concentrations of at least 1280 µg/mL or ten times the highest concentration to be tested, whichever is greater.

v. **Use of Solvents other than water:** Some drugs must be dissolved in solvents other than water as mentioned below. Such drugs should be.dissolved at concentrations at least 100 times higher than the highest desired test concentration. When such solvents are used a series of dilutions at 100 times the final concentration should be prepared from the antifungal stock solution in the same solvent. Each intermediate solution should then be further diluted to final strength in the test medium. For example to prepare for a broth macrodilution test series containing a water-insoluble drug that can.be dissolved in DMSO for which the highest desired test concentration is 6 µg/mL first weigh 4.8 mg (assuming100% potency) of the antifungal powder and dissolve it in 3.0 mL DMSO. This will provide a stock solution at 1,600 µg/mL. Next, prepare further dilutions of this stock solution in DMSO (See Tables 1 and 2). The solutions in DMSO will be diluted 1:50 in test medium and a further two fold when inoculated, reducing the final solvent concentration to 1% DMSO at this concentration (without drug) should be used in the test as a dilution control. The example above assumes 100% potency of the antifungal powder. If the potency is different, the calculations as mentioned above should be applied.

vi. **Filtration:** Normally, stock solutions do not support contaminating microorganisms and they can be assumed to be sterile. If additional assurance of sterility is desired, they are to be filtered through a membrane filter paper, asbestos, or sintered glass filters,which may adsorb appreciable amounts of certain antifungal agents, are not to be used. Whenever filtration is used, it is important that the absence of adsorption by appropriate assay procedures is documented.

vii. **Storage:** Small volumes of the sterile stock solutions are dispensed into sterile polypropylene or polyethylene vials, carefully sealed, and stored (preferably at -60°C or below but never at a temperature greater than -20°C). Vials are to be removed as needed and used the same day. Any unused drug is to be discarded at the end of the day. Stock solutions of most antifungal agents can be stored at -60°C or below for six months or more without significant loss of activity. In all cases, any directions provided by the drug manufacturer are to be considered as a part of these general recommendations and should supersede any other directions that differ. Any significant deterioration of.an. antifungal agent may be ascertained by comparing with the quality control strains.

viii. **Number of concentrations tested:** The concentrations to be tested should encompass the breakpoint concentrations and the expected results for the quality control strains. Based on previous studies, the following drug concentration ranges should be used: amphotericin B, 0.0313.to 16 µg/mL; flucytosine, 0.125 to 64 µg/mL; ketoconazole,

0.0313 to 16 µg/mL; itraconazole, posaconazole, voriconazole 0.0313 to 16 µg/mL; fluconazole 0.125 to.64 µg/mL; and anidulafungin, caspofungin and micafungin 0.015 to 8 µg/mL.

Broth Microdilution test procedure

i. Medium: A completely synthetic medium, RPMI 1640 (with glutamine, without bicarbonate, and with phenol red as pH indicator) should be used.

ii. Buffers: Media should be buffered to a pH of 7.0 ± 0.1 at 25°C using MOPS [3-(N-morpholino) propanesulfonic acid] (final concentration 0.165 mol/L for pH 7.0) . The pH of each batch of medium is to be checked with a pH meter when the medium is prepared; the pH should be between 6.9 and 7.1 at room temperature (25°C). MIC performance characteristics of each batch of broth are evaluated using a standard set of quality control organisms.

iii. Water Soluble antifungal agents: When two fold dilutions of a water-soluble antifungal are to be used, they may be prepared volumetrically in broth (Table – 2).

The procedure for antifungals that are not soluble in water is different from that for water-soluble.agents and is described below. When running a small number of tests, consulting the schedule in Table 2 is recommended. The total volume of each antifungal dilution to be prepared depends on the number of tests to be performed. Because 0.1 mL of each antifungal drug dilution will be used for each test, 1.0 mL will be adequate for about nine tests, allowing for pipetting. A single pipette is used for measuring all diluents and then for adding the stock antifungal solution to the first tube. A separate pipette is used for each remaining dilution in that set because there will be a 1:2 dilution of the drugs.

Table-2 Scheme for Preparing Dilutions of Water Soluble Antifungal Agents to be used in Broth Dilution Susceptibility Tests

Drug - Starting Concentration (µg/ml)	Stock 5120	2	4 160	8	16	32 20	64	128	256 2.5	511	Remarks
		2X	4X	2X	4X	8X	2X	4X	8X	2X	
Tube #	TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5	TUBE 6	TUBE 7	TUBE 8	TUBE 9	TUBE 10	
Source	From Stock 1.0	From Tube 1 1.0	From Tube 1 1.0	From Tube 3 1.0	From Tube 3 0.5	From Tube 3 0.5	From Tube 6 1.0	From Tube 6 0.5	From Tube 6 0.5	From Tube 9 1.0	Step 1 Row 1
Add DRUG Amount (ml) +		+	+	+	+	+	+	+	+	+	
Add Solvent RPMI (ml)	7.0	1.0	3.0	1.0	1.5	3.5	1.0	1.5	3.5	1.0	
Intermediate Drug Concentration (µg/ml)	640	320	160	80	40	20	10	5	2.5	1.25	
Add Drug from Tube Row 1 Above (ml) +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	Step 2 Row 2 5X (1:4)
RPMI 1640 (ml)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	
Final Concentration at 1:5 (µg/ml)	128	64	32	16	8	4	2	1	0.5	.25	2X
From Row 2 add Drug to Micro titer plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 3 (1:1)
Add Inoculum to Plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 4 (T.Vol 0.2ml)
FINAL DRUG CONC in Well at 1: 100 (µg/ml)	64	32	16	8	4	2	1	0.5	0.25	0.125	

iv. Water Insoluble antifungal agents: For antifungal agents that cannot be prepared as stock solutions in water, such as ketoconazole, amphotericin B, or itraconazole, a dilution series

of the agent should be prepared first at 100x final strength in dimethyl sulfoxide (DMSO) (Table-3). Each of these non-aqueous solutions should now be diluted tenfold in RPMI 1640 broth. For example, if a dilution series with final concentrations in the range 16 µg/mL to 0.0313 µg/mL is desired, a concentration series from 1,600 to 3.13 µg/mL should have been prepared first in DMSO. To prepare 1 mL volumes of diluted antifungal agent (sufficient for 10 tests), first pipette 0.9 mL volumes of RPMI 1640 broth into each of 11 sterile test tubes. Now, using a single pipette, add 0.1 mL of DMSO alone to.one 0.9 mL lot of broth (control medium), then 0.1 mL of the lowest (3.13 µg/mL) drug concentration in DMSO, then 0.1 mL of the 6.25 µg/mL concentration and continue in sequence up the concentration series, each time adding 0.1 mL volumes to.0.9 mL broth. These volumes can be adjusted according to the total number of tests required.

v. Inoculum Preparation: The steps for preparation of inoculum are as follows:

1) All organisms should be sub-cultured from sterile vials onto Sabouraud dextrose agar or peptone dextrose agar and passaged at least twice to ensure purity and viability. The incubation temperature throughout must be 35°C.

2) The inoculum should be prepared by picking five colonies of ~ 1 mm in diameter from 24 hour old culture of *Candida* species or 48 hours old cultures of *C. neoformans*. The colonies should be suspended in 5 mL of sterile 0.145 mol/L saline (8.5 g/L NaCl; 0.85% saline).

3) The resulting suspension should be vortexed for 15 seconds and the cell density adjusted with a spectrophotometer by adding sufficient sterile saline to increase the transmittance to that produced by a 0.5 McFarland standard, approximately 0.09-1.0 OD at 530 nm wavelength. This procedure will yield a yeast stock suspension of 1 x 10^6 to 5 x 10^6 cells per mL. A working suspension is made by a 1:50 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 broth medium which results in 5 x 10^2 to 2.5 x 10^3 cells per mL.

Table 3: Scheme for Preparing Dilution Series of Water-Insoluble Antifungal Agents to be used in Broth Dilution Susceptibility Tests

Drug - Starting Concentration (µg/ml)	1,600	2	4	8 200	16	32	64 25	128	256	511	Remarks
Tube #	TUBE 1 (Stock) (100 X)	2X TUBE 2	4X TUBE 3	8X TUBE 4	2X TUBE 5	4X TUBE 6	8X TUBE 7	2X TUBE 8	4X TUBE 9	8X TUBE 10	
Source	From Tube 1	From Tube 1	From Tube 1	From Tube 1	From Tube 4	From Tube 4	From Tube 4	From Tube 7	From Tube 7	From Tube 7	Step 1
Add DRUG Amount (ml)	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	Row 1
+		+	+	+	+	+	+	+	+	+	
Add Solvent DMSO (ml)	-	0.5	1.5	3.5	0.5	1.5	3.5	0.5	1.5	3.5	
Intermediate Drug Concentration (µg/ml)	1,600	800	400	200	100	50	25	12.5	6.25	3.13	
Add Drug from Tube Row 1 Above (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 2
+	+	+	+	+	+	+	+	+	+	+	Row 2
RPMI 1640 (ml)	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	(1:50)
Final Concentration at 1:50 (µg/ml)	32	16	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.0625	(2X)
From Row 2 add Drug to Micro titer plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 3 (1:1)
Add Inoculum to Plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 4 (T.Vol 0.2ml)
FINAL DRUG CONC in Well at 1: 100 (µg/ml)	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.0313	

vi. **Incubation:** With the exception of *C. neoformans*, tubes are incubated (without agitation) at 35°C for 24 to 48 hours in ambient air. When testing *C. neoformans*, tubes should be incubated for a total of 70 to 74 hours before determining results.

vii. **Reading Results:** The MIC is the lowest concentration of an antifungal that substantially inhibits growth of the organism as detected visually. The amount of growth in the tubes containing the agent is compared with the amount of growth in the growth control tubes (no antifungal agent) used in each set of tests as follows:

Amphotericin B: For amphotericin B end points are typically easily defined and the MIC is read as the lowest drug concentration that prevents any discernible growth. Trailing end points with amphotericin B are not usually encountered.

Flucytosine and azoles antifungal: For fluconazole end point is typically less sharp and may be a significant source of variability. A less stringent end point (slight turbidity is allowed above the MIC) has improved inter-laboratory agreement and also discriminates between putatively susceptible and resistant isolates. When turbidity persists, it is often identical for all drug concentrations above the MIC. The amount of allowable turbidity can be estimated by diluting 0.2 mL of drug-free control growth with 0.8 mL of media, producing an 80% inhibition standard. Even dispersion of clumps that can become evident after incubation can make end-point determination more reproducible.

Interpretation of Results: Interpretive breakpoints have been established at present only for some organism-drug combinations. The clinical relevance of testing other organism-drug combinations remains uncertain, but the relevant information can be summarized as follows:

Amphotericin B: Experience to date indicates that amphotericin B MICs for *Candida* spp. isolates are tightly clustered between 0.25 and 1.0 µg/mL.

Fluconazole: Interpretive breakpoints for *Candida* spp. and fluconazole have been established. These interpretive breakpoints are not applicable to *C. krusei*, and thus identification to the species level is required in addition to MIC determination.

Itraconazole: Interpretive breakpoints for *Candida* spp. and itraconazole have been established. The importance of proper preparation of drug dilutions for this insoluble compound cannot be over emphasized. Use of the incorrect solvents or deviation from the dilution scheme suggested in Table 3 can lead to substantial errors due to dilution artifacts.

New Triazoles: Posaconazole and voriconazole MICs vary between 0.03 and 16 µg/mL with the majority of isolates inhibited by <1 µg/mL.

viii. **Broth Microdilution:** The broth microdilution test is performed by using sterile, disposable, multiwell microdilution plates (96 U- shaped wells). The 2x drug concentrations are dispensed into the wells of rows 1 to 10 of the microdilution plates in 100 mL volumes with a multichannel pipette. Row 1 contains the highest (either 64 or 16 µg/mL) drug concentration and row 10 contains the lowest drug concentration (either 0.12 or 0.03 µg/mL). These trays may be sealed in plastic bags and stored frozen at -70°C for up to 6 months without deterioration of drug potency. Each well of a microdilution tray is inoculated on the day of the test with 100 µL of the corresponding 2x diluted inoculum suspension, which brings the drug dilutions and inoculum densities to the final concentrations mentioned above. The growth control wells contain 100 µL of sterile drug-free medium and are inoculated with 100 µL of the corresponding diluted (2X) inoculum suspensions. The QC organisms are tested in the same manner and are included

each time an isolate is tested. Row 11 of the microdilution plate should be used to perform the sterility control (drug-free medium only).

The microdilution plates are incubated at 35°C and observed for the presence or absence of visible growth. The microdilution wells are scored with the aid of a reading mirror, the growth in each well is compared with that of the growth control (drug-free) well. A numerical score, which ranges from 0 to 4, is given to each well using the following scale: 0, optically clear; 1, slightly hazy; 2, prominent decrease in turbidity; 3, slight deduction in turbidity; and 4, no reduction of turbidity. For isolates in which clumping hinders applying these definitions, dispersion of the yeast suspension by pipetting, vortexing or other techniques can help. The MIC for amphotericin B is defined as the lowest concentration in which a score of 0 (optically clear) is observed and for 5-FC and the azoles, as the lowest concentration in which a score of 2 (prominent decrease in turbidity) is observed. Prominent decrease in turbidity corresponds to approximately 50% inhibition in growth as determined spectrophotometrically. The microdilution MICs read at 48 hours (72 hours for most *C. neoformans*) provide the best agreement with the reference broth macrodilution method.

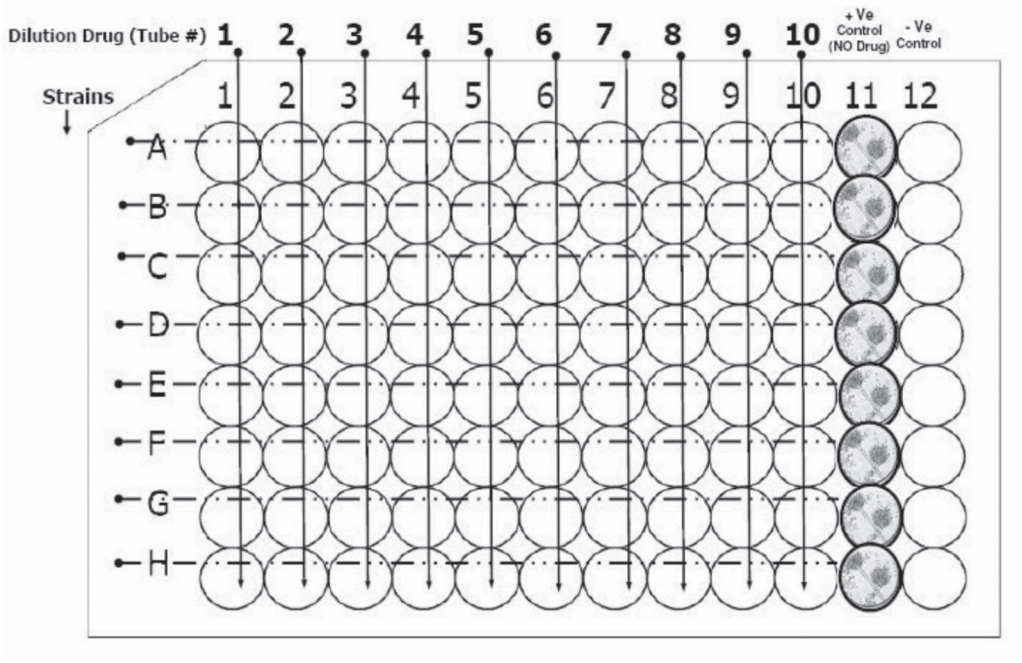

Fig. 2 Suggested Plating Scheme for Broth Microdilution Test;

ix. Impact of Time Reading (24 Hours versus 48 Hours): For *Candida* spp. an end point reading at 48 hours is recommended. For most isolates, the difference between readings at 24 hours versus 48 hours is minimal and will not alter the interpretative category (i.e., does not change whether the isolate would be categorized as "susceptible" or "resistant"). However, recent work has begun to include 24-hour readings, because (a) MICs can often be read at 24 hours; and (b) readings taken at 24 hours may be more clinically relevant for some isolates. Both 24-hour and 48-hour microdilution MIC ranges are provided for the two QC strains and eight systemic antifungal agents (Table 4).

x. Quality Control:

Growth Control: Each broth macrodilution series should include a growth control of basal medium without antifungal agent to assess viability of the test organisms. With the broth

tests, the growth control also serves as a turbidity control for reading end points.

Purity Control: A sample of each inoculum is streaked on a suitable agar plate and incubated overnight to detect mixed cultures and to provide freshly isolated colonies in the event retesting proves necessary.

End Point Interpretation Control: End point interpretation is monitored periodically to minimize variation in the interpretation of MIC end points among observers. All laboratory personnel who perform these tests should read a selected set of dilution tests independently. The results are recorded and compared to the results obtained by an experienced reader.

Quality Control Strains: Ideal reference strains for quality control of dilution tests have MICs that fall near the middle of the concentration range tested for all antifungal agents; e.g. an ideal control strain would be inhibited at the fourth dilution of a seven dilution series, but strains with MICs at either the third or fifth dilution would also be acceptable. Table 5 lists expected ranges for strains found to be acceptable as quality control strains. Also shown are additional strains that can be useful for conducting reference studies.

Table 4: Recommended MIC limits for Two Quality Control Strains for Broth Microdilution Procedures - MIC (µg/ml) ranges for microdilution tests

Organism	**Antifungal Agent**	**Range**	**Mode**	% **within**	**Range**	**Mode**	%
		24 hours		**Range**	**48 hours**		**within Range**
Candida parapsilosis ATCC®22019	Amphotericin B	0.25-2.0	0.5	97	0.5-4.0	2.0	92
	Fluconazole	0.06-0.25	0.12	99	0.12-0.5	0.25	98
	Itraconazole	0.12-0.5	0.25	96	0.12-0.5	0.25	98
	Voriconazole	0.016-0.12	0.06	100	0.03-0.25	0.06	100
	Posaconazole	0.06-0.25	0.12	97	0.06-0.25	0.12	99
Candida krusei ATCC®6258	Amphotericin B	0.5-2.0	1.0	100	1.0-4.0	2.0	100
	Fluconazole	8.0-64	16	100	16-128	32	100
	Itraconazole	0.12-1.0	0.5	96	0.25-1.0	0.5	100
	Voriconazole	0.06-0.5	0.25	98	0.25-1.0	0.5	100
	Posaconazole	0.06-0.5	0.25	100	0.12-1.0	0.5	99

Table 5: Recommended MIC limits for Four Reference Strains for Broth Microdilution Procedures

Organism	Purpose	Antifungal Agent	MIC Range (µg/mL)	% of MICs within Range
Candida parapsilosis ATCC®22019	QC	Amphotericin B	0.25-1.0	99.1
		Fluconazole	2.0-8.0	99.1
		Itraconazole	0.06-0.25	99.0
Candida krusei ATCC®6258	QC	Amphotericin B	0.25-2.0	99.5
		Fluconazole	16-64	99.1
		Itraconazole	0.12-0.5	94.0
Candida albicans ATCC®90028	Reference	Amphotericin B	0.25-1.0	99.5
		Fluconazole	0.25-1.0	95.9
Candida parapsilosis ATCC®90018	Reference	Amphotericin B	0.5-2.0	96.4
		Fluconazole	0.25-1.0	98.2
Candida tropicalis ATCC®750	Reference	Amphotericin B	0.5-2.0	93.7
		Fluconazole	1.0-4.0	95.5

Note: ATCC is a registered trademark of the American Type Culture Collection.

Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts

Introduction

The method described here is intended for testing *Candida* species. This method does not currently encompass any other genera and has not been used in studies of the yeast form of dimorphic fungi, such as *Blastomyces dermatitidis* or *Histoplasma capsulatum*. The method described herein must be followed exactly to obtain reproducible results.

Zone interpretation criteria as per M44/A2 protocol of CLSI are available for fluconazole, voriconazole, and caspofungin and recommended quality control ranges for caspofungin, fluconazole, posaconazole and voriconazole.

1. Reagents for the Disk Diffusion Test

i. Mueller-Hinton Agar + 2% Glucose and 0.5 µg/mL Methylene Blue Dye (GMB) Medium - Of the many agar media available, supplemented Mueller-Hinton agar to be a good choice for routine susceptibility testing of yeasts.

ii. pH of Mueller-Hinton Agar + 2% Glucose and 0.5 µg/mL Methylene Blue Dye Medium - The pH of each batch of prepared Mueller-Hinton agar should be checked. The agar medium should have a pH between 7.2 and 7.4 at room temperature after gelling. The pH can be checked by one of the following means:

- Macerate a sufficient amount of agar to submerge the tip of a pH electrode.
- Allow a small amount of agar to solidify around the tip of a pH electrode in a beaker or cup.
- Use a properly calibrated surface electrode.

iii. Moisture on Agar Surface - If excess surface moisture is present, the agar plates should be dried in an incubator or laminar flow hood with the lids ajar until the excess moisture has evaporated (usually 10 to 30 minutes) . The surface should be moist, but with no droplets on the agar surface or the petri dish cover.

iv. Storage of Antimicrobial Disks - Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Disks should be stored as follows:

- Refrigerate the containers at 8 °C or below, or freeze at -14°C or below, in a non-frost-free freezer until needed. The disks may retain greater stability if stored frozen until the day of use. Always refer to instructions in the product insert.
- The unopened disk containers should be removed from the refrigerator or freezer 30 to 60 minutes before use so they may equilibrate to room temperature before opening. This procedure minimizes the amount of condensation that occurs when warm air contacts cold disks.
- Once a cartridge of disks has been removed from its sealed packaging, it should be placed in a tightly sealed, desiccated container.
- A disk-dispensing apparatus should be fitted with a tight cover and supplied with an adequate desiccant. The dispenser should be allowed to warm to room temperature before opening. The desiccant should be replaced when the indicator changes color.

- When not in use, the dispensing apparatus containing the disks should always be refrigerated.
- Only disks within their valid shelf life may be used. Disks should be discarded on the expiration date.

v. Turbidity Standard for Inoculum Preparation: To standardize the inoculum density for a susceptibility test, a $BaSO_4$ suspension with turbidity, equivalent to a McFarland standard or its optical equivalent should be used.

2. Procedure for Performing the Disk Diffusion Test

i. Inoculum Preparation: Direct Colony Suspension Method

Steps for preparation of the inoculum are as follows:

(1) All organisms need to be subcultured onto blood agar or Sabouraud dextrose agar to ensure purity and viability. The incubation temperature throughout must be 35 °C (±2 °C).

(2) Inoculum is prepared by picking **five distinct colonies** of approximately 1 mm in diameter from a 24-hour-old culture of *Candida* species. Colonies are suspended in 5 mL of sterile 0.145 mol/L saline (8.5 g/L NaCl; 0.85% saline).

(3) The resulting suspension is vortexed for 15 seconds and its turbidity is adjusted either visually or with a spectrophotometer by adding sufficient sterile saline or more colonies to adjust the transmittance to that produced by a 0.5 McFarland standard at 530 nm wavelength. This procedure will yield a yeast stock suspension of 1×10^6 to 5×10^6 cells per mL and should produce semi-confluent growth with most *Candida* species isolates.

ii. Inoculation of Test Plates

(1) Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the suspension. The swab should be rotated several times and pressed firmly against the inside wall of the tube above the fluid level. This will remove excess fluid from the swab.

(2) The dried surface of a sterile Mueller-Hinton + GMB agar plate is inoculated by evenly streaking the swab over the entire agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar is swabbed.

(3) The lid may be left ajar for three to five minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug-impregnated disks.

NOTE: Variations in inoculum density must be avoided. Never use undiluted overnight broth cultures or other unstandardized inocula for streaking plates.

iii. Application of Disks to Inoculated Agar Plates

(1) Antimicrobial disks are dispensed onto the surface of the inoculated agar plate. Each disk must be pressed down to ensure its complete contact with the agar surface. Whether the disks are placed individually or with a dispensing apparatus, they must be distributed evenly so that they are no closer than 24 mm from center to center. Ordinarily, no more than 12 disks should be placed on a 150-mm plate, or more than

five disks on a 100-mm plate. Because the drug diffuses almost instantaneously, a disk should not be moved once it has come into contact with the agar surface. Instead, place a new disk in another location on the agar. Disk should be placed no less than 10 mm from the edge of the petri dish.

(2) The plates are inverted and placed in an incubator set to 35°C (± 2°C) within 15 minutes after the disks are applied.

3. Reading Plates

Examine each plate after 20 to 24 hours of incubation. If the plate was satisfactorily streaked and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a semi confluent lawn of growth. The plate is held a few inches above a black, nonreflecting background illuminated with reflected light. Measure the zone diameter to the nearest whole millimeter at the point at which there is a prominent reduction in growth. This is highly subjective, and experience results in greater accuracy (trueness). Pinpoint microcolonies at the zone edge or large colonies within a zone are encountered frequently and should be ignored. If these colonies are subcultured and retested, identical results are usually obtained, i.e., a clear zone with microcolonies at the zone edge or large colonies within the zone. Read at 48 hours only when insufficient growth is observed after 24 hours incubation.

4. Interpretation of Disk Diffusion Test Results: Tables 6-10 provides zone diameter interpretive criteria to categorize accurately the levels of susceptibility of organisms to antifungal agents.

Interpretive Categories

Susceptible (S): The susceptible category implies that an infection due to the strain may be appropriately treated with the dose of antimicrobial agent recommended for that type of infection and infecting species, unless otherwise contraindicated.

Susceptible-Dose Dependent (S-DD): The susceptible-dose dependent category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates. Susceptibility is dependent on achieving the maximal possible blood level. This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.

Intermediate (I): The intermediate category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates and/ or available data do not permit them to be clearly categorized as either "susceptible" or "resistant". This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations.

Resistant (R): Resistant strains are those that are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules or when zone diameters have been in a range where clinical efficacy has not been reliable in treatment studies.

Nonsusceptible (NS): The nonsusceptible category includes organisms that currently have only a susceptible interpretive category, but not intermediate, susceptible-dose dependent, or resistant interpretive categories. This category is often given to new antimicrobial agents for which no resistant isolates have yet been encountered.

Zone Diameter Interpretive Criteria

Disk diffusion zone diameters correlate inversely with MICs from standard dilution tests. Table 6-10 lists the zone diameter interpretive criteria.

5. Quality Control

Part of quality management focused on fulfilling quality requirements, which includes operational techniques and activities used to fulfill these requirements.

Reference Strains for Quality Control

To control the precision (repeatability) and accuracy (trueness) of the results obtained with disk diffusion test procedure, several quality control strains should be obtained from a reliable source. The recommended quality control strains include:

- *Candida albicans* ATCC 90028;
- *Candida parapsilosis* ATCC 22019;
- *Candida tropicalis* ATCC 750; and
- *Candida krusei* ATCC 6258.

6. Zone Diameter Quality Control Limits

Acceptable zone diameter quality control limits for quality control strains are listed in Table 6. The overall performance of the test system should be monitored using these ranges by testing the appropriate control strains each day the test is performed or, if satisfactory performance is documented, testing may be done weekly.

7. Frequency of Quality Control Testing

Daily Testing:- When testing is performed daily, for each antimicrobial agent/organism combination, 1 out of every 20 consecutive results may be out of the acceptable range (based on 95% confidence limits, 1 out of 20 random results may be out of control). Any more than 1 out-of-control result in 20 consecutive tests requires corrective action.

Weekly Testing: - Demonstrating Satisfactory Performance for Conversion from Daily to weekly Quality Control Testing

- Test all applicable control strains for 20 consecutive test days and document results.
- To convert from daily to weekly quality control testing, no more than 1 out of 20 zone diameters for each antimicrobial agent/organism combination may be outside the acceptable zone diameter limits in Table 5.

8. Implementing Weekly Quality Control Testing

- Weekly quality control testing may be implemented once satisfactory performance has been documented
- Perform quality control testing once per week and whenever any reagent component of the test (e.g., a new lot of agar plates or a new lot of disks from the same or a different manufacturer) is changed.
- If any of the weekly quality control results are out of the acceptable range, corrective action is required.

- If a new antimicrobial agent is added, it must be tested for 20 consecutive test days and satisfactory performance documented before converting to a weekly schedule. In addition, 20 days of consecutive testing are required if there is a major change in the method of reading test results, such as conversion from manual zone measurements to an automated zone reader.

Corrective Action

i. Out-of-Control Result Due to an Obvious Error:

Obvious reasons for out-of-control results include:

- Use of the wrong disk;
- Use of the wrong control strain;
- Obvious contamination of the strain; or
- Inadvertent use of the wrong incubation temperature or conditions.

In such cases, document the reason and retest the strain on the day the error is observed. If the repeated result is within range, no further corrective action is required.

ii. Out-of-Control Result Not Due to an Obvious Error

Immediate Corrective Action: If there is no obvious reason for an out-of-control result, immediate corrective action is required.

- Test the antimicrobial agent/organism combination for a total of five consecutive test days. Document all results in question.
- If all five zone diameter measurements for the antimicrobial agent/organism combination are within acceptable ranges, as defined in Table 6, no additional corrective action is necessary.
- If any of the five zone diameter measurements are outside the acceptable range, additional corrective action is required.
- Daily control tests must be continued until final resolution of the problem can be achieved. Additional Corrective Action When immediate corrective action does not resolve the problem; it is likely due to a system error versus a random error. The following common sources of error should be investigated.
- Zone diameters were measured and transcribed correctly.
- The turbidity standard has not expired, is stored properly, meets performance requirements, and was adequately mixed prior to use.
- All materials used were within their expiration date and stored at the proper temperature.
- The incubator is at the proper temperature and atmosphere.
- Other equipment used (e.g., pipettes) are functioning properly.
- Disks are stored desiccated and at the proper temperature.
- The control strain has not changed and is not contaminated.
- Inoculum suspensions were prepared and adjusted correctly.
- Inoculum for the test was prepared from a plate incubated for the correct length of time and in no case was more than 24 hours old.

It may be necessary to obtain a new quality control strain (either from freezer stock or a reliable source) and new lots of materials (including new turbidity standards), possibly from different manufacturers. If the problem appears to be related to a commercial product, the manufacturer should be contacted. It is also helpful to exchange quality control strains and test materials with another laboratory using the same method. Until the problem is resolved, an alternative test method should be used. Once the problem is corrected, documentation of satisfactory performance for another 20 consecutive days is required before returning to weekly quality control testing.

9. Reporting Patient Results When Out-of-Control Tests Occur

Whenever an out-of-control result or corrective action is necessary, careful assessment of whether to report patient results should be made on an individual basis, taking into account if the source of error, when known, is likely to have affected relevant patient results. Options that may be considered include suppressing the results for an individual antimicrobial agent; retrospectively reviewing individual patient or cumulative data for unusual patterns; and using an alternate test method or a reference laboratory until the problem is resolved.

10. Limitations of Disk Diffusion Methods

i. Application to Various Organism Groups

The disk diffusion method described in this document has been standardized for *Candida* species only. For other yeasts, consultation with an infectious disease specialist is recommended for guidance in determining the need for susceptibility testing and interpretation of results. Published reports in the medical literature and current consensus recommendations for therapy of uncommon microorganisms may obviate the need for testing. If necessary, a reference dilution method may be the most appropriate alternative testing method, and this may require submitting the organism to a reference laboratory.

ii. Verification of Patient Results

Multiple test parameters are monitored by following the quality control recommendations described in this standard. However, acceptable results derived from testing quality control strains do not guarantee accurate results when testing patient isolates. It is important to review all of the results obtained from all drugs tested on a patient's isolates prior to reporting the results.

Unusual or inconsistent results should be verified by checking for the following: 1) transcription errors; 2) contamination of the test (recheck purity plates); and 3) previous results on the patient's isolates. If a reason for the unusual or inconsistent result cannot be ascertained, repeat the susceptibility test, verify the species identity, or request a new clinical specimen. Each laboratory must develop its own policies for verification of unusual or inconsistent antimicrobial susceptibility test results.

Table 6: Recommended Quality Control Zone Diameter (mm) Ranges

Antifungal Agent	Disk Content	*C. albicans* ATCC 90028	*C. parapsilosis* ATCC 22019	*C. tropicalis* ATCC 750	*C. krusei* ATCC 6258
Caspofungin	5 µg	18-27	14-23	20-27	19-26
Fluconazole	25 µg	28 - 39	22 - 33	26 - 37	—*
Voriconazole	1 µg	31 - 42	28 - 37	—*	16 - 25
Posaconazole	5 µg	24-34	25-36	23-33	23-31

*Quality control ranges have not been established for these strain/antimicrobial agent combinations, due to their extensive interlaboratory variation during initial quality control studies

Table 7: List of Antimicrobial agents, Zone diameters andMinimal Inhibitory Concentrations (MIC) interpretative criteria for *Candida albicans, Candida tropicalis*

Antimicrobial Agent	Disc Diffusion Zone diameters (mm)			MIC (µg/ml)			
	Susceptible	Intermediate	Resistant	Susceptible	SDD	Intermediate	Resistant
Anidulafungin	-	-	-	≤0.25	-	0.5	≥1
Caspofungin	**≥17**	**15-16**	**≤14**	≤0.25	**-**	0.5	≥1
Micafungin	**≥22**	**20-21**	**≤19**	≤0.25	**-**	0.5	≥1
Fluconazole	**≥17**	**14-16**	**≤13**	≤2	**4**	**-**	≥8
Itraconazole				≤0.125	**0.25-0.5**		≥1
Voriconazole	**≥17**	**15-16**	**≤14**	≤0.125	**0.25-0.5**	**-**	≥1

S-Susceptible; S-DD – Susceptible Dose Dependent; R-Resistant, and NS-nonsusceptible Interpretive categories
† Isolates of *C. krusei* are assumed to be intrinsically resistant to fluconazole. The results of fluconazole susceptibility testing of this species (zone diameter and MIC) should not be interpreted using this scale.

Table 8: List of Antimicrobial agents, Zone diameters and Minimal Inhibitory Concentrations (MIC) interpretative criteria for *Candida glabrata*

Antimicrobial Agent	Disc Diffusion Zone diameters (mm)			MIC (µg/ml)			
	Susceptible	Intermediate	Resistant	Susceptible	SDD	Intermediate	Resistant
Anidulafungin	-	-	-	≤0.125	-	0.25	≥0.5
Caspofungin	-	-		≤0.125	-	0.25	≥0.5
Micafungin	-	-		≤0.06	-	0.125	≥0.25
Fluconazole	-	≥15	≤14	-	≤32	-	≥64
Itraconazole				≤0.125	0.25-0.5		≥1
Voriconazole	-	-	-	-	-	-	-

Table 9: List of Antimicrobial agents, Zone diameters and Minimal Inhibitory Concentrations (MIC) interpretative criteria for *Candida parapsilosis*

Antimicrobial Agent	Disc Diffusion Zone diameters (mm)			MIC (µg/ml)			
	Susceptible	Intermediate	Resistant	Susceptible	SDD	Intermediate	Resistant
Anidulafungin	NA	NA	NA	≤2	NA	4	≥8
Caspofungin	≥13	11-12	≤10	≤2	NA	4	≥8
Micafungin	≥16	14-15	≤13	≤2	NA	4	≥8
Fluconazole	≥17	14-16	≤13	≤2	4	NA	≥8
Itraconazole	NA	NA	NA	≤0.125	0.25-0.5	NA	≥1
Voriconazole	≥15	13-14	≤12	≤0.125	0.25-0.5	NA	≥1

Table 10: List of Antimicrobial Agents, Zone diameters and MIC interpretive criteria for *Candida krusei*

Antimicrobial Agent	Disc Diffusion Zone diameter (mm)			MIC (µg/ml)			
	Susceptible	Intermediate	Resistant	Susceptible	SDD	Intermediate	Resistant
Anidulafungin	NA	NA	NA	≤0.25	NA	0.5	≥ I
Caspofungin	≥ 17	15-16	≤14	≤0.25	NA	0.5	≥ I
Micafungin	≥ 22	20-21	≤19	≤0.25	NA	0.5	≥1
Fluconazole	NA	NA	NA	NA	NA	NA	NA
Voriconazole	≥15	13-14	≤12	≤0.5	1	NA	≥2

Table 11: List of Antimicrobial Agents, Zone diameters and MIC interpretive criteria for *Candida guilliermondii*

Antimicrobial agent	Disc Diffusion Zone diameter (mm)			MIC (µg/ml)			
	Susceptible	Intermediate	Resistant	Susceptible	SDD	Intermediate	Resistant
Anidulafungin	NA	NA	NA	≤2	NA	4	≥8
Caspofungin	≥13	11-12	≤10	≤2	NA	4	≥8
Micafungin	≥16	14-15	≤13	≤2	NA	4	≥8
Fluconazole	NA	NA	NA	NA	NA	NA	NA
Voriconazole	NA	NA	NA	NA	NA	NA	NA

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