I. **PRINCIPLE**:

A. The majority of filamentous fungi recovered in the clinical laboratory belong to the class Hyphomycetes, which includes all of the filamentous, sporulating members of the Fungi Imperfecti except for those species that form pycnidia, acervuli or sporodochia.

B. Zygomycetes, Ascomycetes and Basidiomycetes produce characteristic spores after the processes of plasmogamy, karyogamy, and meiosis. Heterothallism, which requires two mating types, is more common than homothallism; therefore, zygospores, ascospores, and basidiospores are not commonly seen in the clinical laboratory because only one mating type is usually recovered.

C. Many criteria are considered when identifying moulds. Morphology, culture characteristics, temperature tolerance, cycloheximide resistance, dimorphism, nutritional requirements, proteolytic activity, and the ability to hydrolyze urea are criteria used in identifying moulds. Modern classification schemes primarily emphasize conidial ontogeny rather than color, conidial septation or the appearance of growth on natural substrates. Characteristics which vary depending on the media and environmental conditions, or which are subjective, such as color are not consistent enough to be used as the sole criteria in classifying moulds.

1. ZYGOMYCETES

a. Asexual reproduction in the Zygomycetes is typified by a sac-like cell called a sporangium, in which the entire internal content is cleaved into spores. A sporangium having its sporangiospores in a row is called a merosporangium. Reduced sporangia, containing only one or a few sporangiospores are called sporangiola. A columella is a small sterile dome-like area at the apex of the sporangiophore. The term apophysis is applied to any swelling in the sporangiophore where it merges with the columella. Remnants of the sporangial wall which remains attached at the junction of the columella and sporangiophore after the sporangium ruptures or dissolves is called a collarette.

b. Some Zygomycetes form vegetative hyphae similar to runners. These runners, called stolons, and their relationship to origin of the sporangiophore and the location of the rhizoids is a useful feature in delineating several genera. A node is where a stolon touches the substrate, and the arch formed between it and the next node is the internode. When rhizoids develop, they often do so at the node. Some sporangiophores arise at the node, while others arise only from the internode or aerial hyphae. The diameter of the hyphae is a useful characteristic in distinguishing Zygomycetes from other fungi. They typically develop hyphae that are characterized as being sparsely septate, branching irregularly, and having an irregular diameter of 10-15 um.

c. In culture, Zygomycetes are tan to grey to black in color and have a woolly texture. The growth rate is rapid and isolates such as *Rhizopus* and *Syncephalastrum* will fill the culture tube within a few days.

d. Identification Procedure for Zygomycetes

(1) Prepare a tease mount of the Zygomycete within the safety cabinet. Examine the mount using the low and high power objectives of the microscope. Zygomycetes can be differentiated by the Sparsely separated hyphae which is irregular in diameter (10-15 µm).

(2) Examine the culture plate or tube under the dissecting microscope. Rhizoids, stolons, and nodes are easier to evaluate at low power.

I. **PRINCIPLE**: (cont'd)

(3) Prepare a slide culture of the Zygomycete if unable to identify it from the tease mount. Besure to examine both the cover slip and the glass microscope slide.
(4) If the culture remains sterile after subculture, set up a water agar plate to induce sporulation and to rule out the genus *Apophysomyces* or *Sacksenae*.
(5) Temperature tolerance studies should be performed if the organism is suspected of being *Rhizomcos* sp.
(6) Isolates from clinically significant cases that do not match any of the known Qygomycetes should be sent to a reference laboratory for identification or confirmation by mating studies or exuantigen testing. Consult the supervisor for advice.

2. ASCOMYCETES

a. Ascomycetes are characterized by the development of sac-like cells called asci, which usually contain eight ascospores. Asci may form in a specialized fruiting body called an ascocarp. The development of the centrum is very important in distinguishing ascocarp types. The five major types of ascocarps (also referred to as ascoma) are:

(1) Gymnothecium. A gymnothecium is round ascocarp with loosely organized network of hyphae enclosing randomly dispersed asci. There is no natural opening or ostiole in the gymnothecium. Appendages in the form of setae, conidia, etc., may be present or absent, depending upon the species. This type of ascocarp is characteristic of the Gymnoascaceae.
(2) Cleistothecium. A cleistothecium is a round, nonostiolate ascocarp with a peridium consisting of a well organized membrane-like layer of cells that enclose randomly dispersed asci.
(3) Perithecium. A perithecium is a round to flask-shaped ascocarp that typically has a distinct ostiole from which ascospores can escape. The asci are either arranged in a hymenium or in a basal bush.
(4) Apothecium. An apothecium is an open, disk- to cup-shaped ascocarp that may be sessile or stalked, with asci developing on the exposed hymenium. Apothecia are not to be expected in a clinical laboratory.
(5) Ascostroma. An ascostroma is an ascocarp in which a cavity is either dissolved or formed via compression in a stroma, after which the asci are formed within the cavity (locule). The asci in an ascostroma are bitunicate. Ascostromata are not likely to be seen in a clinical laboratory. When present they may be mistaken for perithecia.

b. Identification Procedure for Ascomycetes

(1) Examine the colony for macroscopic structures such as ascocarps. A dissecting microscope is useful in observing ascocarps.
(2) When ascocarps are mature, use a dissecting needle to pick several ascocarps at various stages of maturity and mount them in lactophenol. Place a coverslip over the preparation and examine with a light or phase-contrast microscope.
(3) Asci and ascospores can be released from the ascocarp by gently tapping the coverslip with a pencil. Asci of many genera are evanescent (dissolve at maturity). Therefore, it will be necessary to examine young, immature ascocarps to study ascus morphology in these isolates.

I. **PRINCIPLE: (cont'd)**

3. BASIDIOMYCETES

a. Basidiomycetes are unique in that their vegetative cells are normally dikaryotic (n + n). Most Basidiomycetes maintain the dikaryotic state via clamp connections and special septa called dolipore septa, which at times prevent the migration of nuclei from one cell to the next. A clamp connection is a specialized hypha-bridge that permits the simultaneous mitosis of two nuclei in such a position that the n + n nuclear condition is maintained within each cell of a dikaryotic hypha. As the basidium enlarges, karyogamy occurs, resulting in a zygote nucleus. The diploid nucleus undergoes meiosis and forms four haploid nuclei. Typically, four sterigmata begin to develop from the top of the basidium. The tips enlarge, forming the basidiospore initials. A haploid nucleus migrates through each sterigma into the developing basidiospore. The spores become sealed at their bases, and as they mature, a droplet of water accumulates at their bases. Once the droplet reaches a certain size, each basidiospore is forcibly discharged from its sterigma. Except for poisonous mushrooms, fungi of medical importance rarely produce basidia and basidiospores in basidiocarps.

b. For all practical purposes, fungi which can be proven to be basidiomycetous are rarely seen in the clinical laboratory since most isolates are heterothallic. These fungi can be recognized by the presence of clamp connections, or in the case of species of Rhizoctonia and Sclerotium, by abundant sclerotia in culture. Many, of the Mycelia Sterilia isolated in the clinical laboratory are isolates of heterothallic Basidiomycetes.

4. MYCELIA STERILIA

Mycelia Sterilia is a form-order that contains the filamentous fungi that remain sterile despite attempts to induce the formation of conidia or spores. Sterile isolates represent species of fungi that simply are not producing conidia, spores, pycnidia, ascocarps or basidiocarps because of compatibility systems, the lack of appropriate environmental and nutritional needs, or both. In rare instances, these fungi are opportunistic pathogens of humans. If an isolate is suspected of causing disease, it is important to try to induce the formation of conidia, spores or fruiting bodies so it can be identified. There is no universal medium or set of environmental conditions that will stimulate conidiogenesis or sporogenesis. Various media and techniques must be tried until the correct combination of variables are found. Since most Mycelia Sterilia are not significant isolates, it is not practical to expend much time and material attempting to induce sporulation. These isolates should be tested for resistance to cyclohexamide and the ability to grow at 35-37^{o}C.

5. FUNGI IMPERFECTI

a. Imperfect fungi are fungi in which the perfect stages (teleomorphs) are not known. In mycology, there are two independent classification systems; one for sexual stages or teleomorphs and a second for asexual forms or anamorphs. The entire fungus, which includes all of its anamorphs and teleomorph is referred to as the holomorph. Mycologists classify teleomorphs to reflect phylogeny in order to determine relatedness of different groups. Anamorphs are classified in groups which do not show or imply phylogenetic relationships, called form-phyla, form-classes, form-orders, form-families, form-genera, and form-species.

I. **PRINCIPLE**: (cont'd)

Some of the imperfect fungi can produce sexual structures under appropriate conditions. When the teleomorph is present, the proper name is based upon the sexual stage. The name for pleomorphic fungi, which may have many different anamorphs is based upon the most distinctive, conspicuous, often encountered, and stable anamorph.

b. The filamentous members of the Fungi Imperfecti are distinguished on the basis of the presence or absence of fruiting structures. The class Coelomycetes contains two orders, the Melanconiales, which produce acervuli and the Sphaeropsidales, which produce pycnidia. The second class is the Hyphomycetes. The majority of filamentous fungi recovered in the clinical laboratory belong to the class Hyphomycetes. Many classification schemes have been developed for the Hyphomycetes. Keys based on conidial ontogeny provide less subjective criteria than keys based on the arrangement and color of the conidiophores and conidia.

c. Identification Procedure for the Hyphomycetes

(1) Prepare a tease mount of the fungus in lactophenol within the biological safety cabinet.
(2) Examine the mount microscopically.
(3) Observe structures such as conidia, conidiophores; their morphology, arrangement and ontogeny. If unable to evaluate these structures with a tease mount, prepare a slide culture. Use of slide cultures is prohibited when working with suspected isolates of *Coccidioides*, *Blastomyces*, *Histoplasma*, *Paracoccidioides,* and *Xylohypha bantiana*.
(4) Using the tease mount or slide culture preparation, attempt to identify the mould to the genus level by using the criteria used in this section.
(5) In most occasions, the organism will be identified from the primary inoculation plates. If the organism has not sporulated, proceed as follows:

(a) For molds from sterile sites or colony morphology resembling a dimorphic (colonies show up after 10 days of incubation or colonies are yeast-like, restricted growth, and or white leathery-like), perform cychlohexamide resistance, confirmation of dimorphism, or DNA probe for the organism that it resembles.
(b) Subculture to Potato Dextrose Agar (PDA) plates and incubate at 25-29°C. Repeat tease mounts or Scoth tapes as needed. Depending on colony morphology and microscopic morphology, set up temperature tolerance studies and/or other tests that may speed up the identification.
(c) If the mould remains sterile after 3 weeks of incubation, consult with supervisor for steps to follow.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION** :

A. Colony Characteristics

1. To evaluate colony characteristics of filamentous fungi, it is necessary to subculture the fungus to the same media that the colony descriptions are based upon. Malt agar, potato dextrose agar, or Czapek-Dox-solution agar are some that have been used by mycologists to evaluate colony characteristics.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

2. Visual examination of the colony will rapidly reveal important data concerning color, texture, diffusible pigments, exudates, growth zones, aerial and submerged hyphae, growth rate, colony topography, and macroscopic structures such as ascocarps, pycnidia, sclerotia, sporodochia, and synnemata.
3. Use of a dissecting microscope will help bridge the gross colony characteristics and microscopic observations.

B. Confirmation of Dimorphism

Dimorphic fungi have the ability to grow vegetatively at 25ºC as moulds and at 37ºC, (in tissue or on special media) as yeasts, spherules or enlarging endospores. These fungi include *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Coccidioides immitis*, *Penicillium marneffei*, and *Sporothrix schenckii*. If a mould is suspected of being one of these dimorphic fungi, it is necessary to convert the mould phase to the tissue phase to confirm the identification, or demonstrate the presence of genus-species specific exoantigens are present by the immunodiffusion technique. Total conversion of an isolate is not necessary for demonstrating dimorphism. Conversion can be very difficult with some isolates, requiring multiple subcultures and special media. DNA probes are commercially available for the identification of some of the dimorphic fungi. Refer to the DNA proble procedures for details.

1. Procedure for Mould-to-Yeast Conversion

a. Allow 2 tubes of the appropriate conversion medium to warm to room temperature.

Suspected Fungus	Media	Incubation Temperature
Blastomyces dermatitidis	BHI with blood agar	35 - 37°C
Histoplasma capsulatum	BHI agar plus glutamine or BHI with blood agar	35 - 37°C
Paracoccidioides brasiliensis	BHI agar plus 20% blood	35 - 37°C
Penicillium marneffei	BHI agar plus 20% blood	35 - 37°C
Sporothrix schenckii	BHI agar plus 20% blood	35 - 37°C, 5-10% CO_2

b. Perform all procedures within a biological safety cabinet.
c. With a long-handled inoculating needle, remove a small portion of the isolate and transfer it to two tubes of medium. Incubate one tube at 37°C and one at 25°C.
d. Check the media weekly for areas of yeast-like growth. Several weeks may be required for conversion.
e. Prepare tease mounts of the yeast-like growth and examine for the presence of hyphae and yeast cells.
f. Positive: Mould conversion to typical yeast morphology. Negative: Mould not converted to yeast phase.
g. Quality Control: A known culture is run con-currently with each conversion to verify the performance of the culture media used in the conversion process.

C. Confirmation of *Coccidioides immitis*

Isolates suspected of being *C. immitis* should be confirmed by performance of the exoantigen test or using DNA probes. See the procedure for performance of the exoantigen test.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

D. Cycloheximide Resistance

1. Determining the resistance of isolates to cycloheximide (0.5 mg/ml) is useful when screening cultures for *Blastomyces dermatitidis*, *Coccidioides immitis*, *Epidermophyton floccosum*, *Histoplasma capsulatum*, *Microsporum* spp., *Paracoccidioides brasiliensis*, *Sporothrix schenckii*, and *Trichophyton* spp. All of these fungi will grow in the presence of cycloheximide at 30°C or less, while fungi such as *Absidia*, *Aspergillus*, *Mucor*, *Rhizopus*, *Scedosporium*, and many more are inhibited by cycloheximide.

2. Procedure

   a. Remove a small portion of the mould colony with an inoculating needle from midway between the center and edge of the colony. Inoculate one plate of Mycosel and one plate of SAB.
   b. Incubate the cultures at 30°C for 7-10 days.
   c. Read and record results.

3. Results

   a. Resistant - growth on medium plus cycloheximide and SAB.
   b. Sensitive - growth on SAB, no growth on medium plus cycloheximide.
   c. Repeat test - no growth on SAB nor medium plus cycloheximide.

4. Quality Control

   A cycloheximide sensitive isolate is run con-currently with each procedure to confirm the cycloheximide activity in the medium plus cycloheximide.

E. Forcibly Discharged Conidia or Spores

1. Fungi such as members of the genera *Basidiobolus*, *Conidiobolus*, *Sporobolomyces*, and *Tilletiopsis* have the ability to produce ballistospores.

2. Procedure

   a. Inoculate a plate of potato dextrose agar (PDA) with the isolate. Tape the plate to a second uninoculated plate of PDA so that the media surfaces face each other directly. Incubate with the inoculated surface on top at 20-25°C for 2-3 days.
   b. If ballistospores are formed, new growth in the form of a mirror image of the original colony will be present on the uninoculated surface.

F. *In Vitro* Hair Test

1. A number of dermatophytes have the ability to penetrate hair in vitro. The test is particularly useful in distinguishing atypical isolates of Trichophyton mentagrophytes from T. rubrum.

2. Procedure

   a. Place several sterile hairs in a sterile glass Petri dish. Hair from a blond child is preferred.
   b. Add 25 ml of sterile water and 0.1 ml of sterile yeast extract to the Petri dish.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

c. Transfer a small amount of the fungus colony to the hairs in the Petri dish.
d. Incubate at 25°C and examine weekly for up to 4 weeks.
e. Examine hairs by placing a few hairs in a drop of lactophenol on a microscope slide. Place a cover glass over the preparation and examine microscopically. It will be necessary to examine several hairs before concluding a negative result.

3. Results

a. Positive - Presence of perforations (conical or wedge-like holes) in the hair.
b. Negative - Absence of perforations in the hair.

4. Quality Control - Known isolates are run concurrently as controls.

a. Positive - *T. mentagrophytes*
b. Negative - *T. rubrum*

G. Nutritional Studies

1. Nutritional studies can be extremely valuable in identifying some species of moulds, especially dermatophytes. These studies test the isolate's requirements for selected vitamins.

2. Procedure for Vitamin Studies

a. Prepare the inoculum by subculturing isolates to be tested to Sabouraud dextrose agar for 7-14 days at 25°C.
b. With an inoculating needle, transfer a small portion of the colony that is approximately 1 mm in diameter to the test media. Avoid transferring agar along with the vegetative inoculum.
c. Incubate at 25°C for 7-14 days. When testing suspected isolates of *T. concentricum*, *T. schoenleinii*, and *T. verrucosum*, incubate at 37°C for 7-14 days.
d. Read the tests and compare the relative amounts of growth in each independent set of tests. Trace amount of growth is +, whereas the maximum amount of growth is 4+.

3. Test Media Components

Medium No.	Components
1	Basal medium only (casein)
2	Basal medium + inositol
3	Basal medium + inositol + thiamine
4	Basal medium + thiamine
5	Basal medium + nicotinic acid
6	Ammonium nitrate
7	Ammonium nitrate + histidine

Nutritional Agars for Differentiation of *Trichophyton* species (commerically available from Difco, Detroit).

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

4. Casein agar

casein, 10% acid hydrolyzed	25.0 ml
glucose	40.0 gm
Mg $S0_4$	0.1 gm
KH_2 PO_4	1.8 gm
agar	20.0 gm
distilled H_2O	q.s. 1000 ml
adjust to pH 6.8	

5. Ammonium nitrate agar - Same as casein agar except substitute 1.5 gm NH_4N0_3 for casein, 10% acid hydrolzyed.

6. Test media

   a. Thiamin - casein agar: 2 ml stock thiamin solution in 100 ml melted casein agar. Final thiamin concentration 0.2 µg/ml.
   b. Inositol-casein agar: 2 ml stock inositol solution in 100 ml melted casein agar. Final inositol concentration 50 µg/ml.
   c. Thiamin-inositol-casein agar: 2 ml of each stock thiamin and inositol solution to 100 ml melted casein agar.
   d. Nicotinic acid-casein agar: 2 ml of stock nicotinic acid solution to 100 ml melted casein agar.
   e. Histidine-ammonium nitrate agar: 2 ml of stock histidine solution to 100 ml melted ammonium nitrate agar.

7. Stock vitamin solutions

a.	Thiamin	thiamin hydrochloride	10.0 mg
		distilled H_20	1000 ml
b.	Inositol	inositol	250.0 mg
		distilled H_20	1000 ml
c.	Nicotinic acid	nicotinic acid	10.0 mg
		distilled H_20	1000 ml
d.	Histidine	l-histidine	150.0 mg
		distilled H_20	1000 ml

8. Results

T. verrucosum	84% of isolates require thiamine and inositol, 16% require only thiamine.
T. concentricum	50% of isolates do not require vitamins; 50% have enhanced growth in the presence of thiamine.
T. tonsurans	growth is greatly enhanced in the presence of thiamine.
T. violaceum	growth is greatly enhanced in the presence of thiamine.
T. equinum	100% of isolates require nicotinic acid for growth.
T. megninii	requires histidine for growth.

9. Quality Control - Known isolates of *T. verrucosum* and *T. tonsurans* will be run concurrently.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

H. Rice Grain Test

1. The sterile rice grain test is useful in distinguishing atypical isolates of *Microsporum canis* from *M. audouinii*.

2. Procedure

   a. With a long-handled inoculating needle, transfer a small portion of the isolate to be tested to a flask containing sterile rice grains.
   b. Incubate the flask at 30°C and examine for growth for 8-10 days.

3. Results

   a. Positive - Rapid growth on rice grains, typically produce many conidia and a bright yellow pigment; *M. canis*.
   b. Negative - Absence of growth with or without a brown discoloration of the rice grains at the site of inoculation; M. *audouinii*.

4. Quality Control - Known isolates of M. canis and M. audouinii are run concurrently with each rice grain test.

I. Lactophenol Mounts

1. Tease Mount Procedure

   a. A tease mount is the most common and quickest technique used to mount fungi for microscopic examination. Since the mould's growth is teased apart with dissecting needles, conidia or spores may be dislodged from the conidiogenous or sporogenous cells. It may be necessary to use the slide culture technique if the identification cannot be made from the tease mount.
   b. Procedure

      (1) Place a drop of lactophenol just off center on a clean microscope slide.
      (2) With a long-handled inoculating needle, gently remove a small portion of growth midway between the colony center and edge. Place the material in the lactophenol.
      (3) With two dissecting needles, gently tease the fungus apart so that it is thinly spread out in the lactophenol.
      (4) Place a coverslip at the edge of the lactophenol and slowly lower it with a sharp pointed object.
      (5) Avoid trapping air bubbles under the coverslip. Remove excess lactophenol from the edges of the coverslip by blotting with a paper towel.
      (6) Seal the edges of the coverslip with fingernail polish to preserve the mount.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

2. Scotch Tape Lactophenol Mount

a. The Scotch tape mount is an easy and fast procedure that is used for the identification of filamentous fungi since most structures will be intact for observation thank to the gummed side of the tape. As with the lactophenol mout, the organism will be immersed in the solution, rendering the organism safe for handling outside of the biological safety hood. Limitations include: the tape will dissolve eventually so that it is not to be used for permanent mounts; the procedure can only be performed on moulds growing from plates.

b. Procedure

(1) Cut a strip of Scotch transparent tape (3M 600, cat. #07457-B) and place ends between thumb and index finger, gummed side out.
(2) Making a loop by closing fingers, open plate with opposite hand and press tape against the colony to identify.
(3) Place a drop of lactophenol on a labeled slide.
(4) Press tape against slide with lactophenol.
(5) Smooth the tape back on the slide by opening fingers and using guaze.
(6) Place another drop of lactophenol on top of the tape.
(7) Place a large 20x40 mm coverslip on top of slide.
(8) Examine the slide under the microscope.

J. Temperature Tolerance

1. Thermotolerance is a useful characteristic that can be used as an aid in the identification of several medically important moulds. Thermotolerance of some medically important fungi include:

Fungus	Upper Growth Limits °C
Aspergillus fumigatus	48-50
C. carrionii	35-36
Fonsecaea pedrosoi	38
Rhizomucor pusillus	45-55
Trichophyton mentagrophytes	37
Wangiella dermatitidis	40
Xylohypha bantiana	42-43

Trichophyton verrucosum has an optimal growth temperature of 37°C. Growth at 37°C will be greater than at 30°C.

2. Procedure

a. Remove small portions of the mould colony from midway between the colony center and edge. Inoculate two plates of media. Media such as potato dextrose agar, cornmeal agar, or Sabouraud dextrose agar are recommended. Plates incubated at elevated temperatures should be sealed with gas-permeable tape to retard dehydration of the media.
b. Incubate one culture at 30°C and one at the elevated temperature.
c. Check cultures periodically for growth.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

3. Results

   a. Positive - growth present at 30°C and at the elevated temperature.
   b. Negative - growth present at 30°C but not at the elevated temperature.
   c. Repeat test if there is no growth at 30°C.

4. Quality Control - This test provides its own internal control. Incubators must be carefully adjusted to the appropriate temperature before incubating the test.

K. Urea Hydrolysis

1. Urea hydrolysis is primarily used to distinguish *Trichophyton mentagrophytes* from *T. rubrum*. The urea hydrolysis test is not definitive but can furnish additional data that may be helpful in identifying atypical isolates of *T. mentagrophytes* and *T. rubrum*. *T. mentagrophytes* is usually urease positive in 7-8 days. *T. rubrum* is usually urease negative, though occasionally urease positive isolates are encountered. It is critical that the purity of the isolate is determined, and that no bacteria are present.

2. Procedure

   a. With a long-handled inoculating needle, remove a portion of growth from midway between the center and the edge of the colony. Inoculate a tube of Christensen's urea agar.
   b. Incubate the inoculated slant and an uninoculated slant at 25-30°C for 8 days. Examine the slants every 2-3 days for a color change.

3. Results

   a. Positive - pink to red color change
   b. Negative - yellow, no color change
   c. Uninoculated control must be negative.

4. Quality Control - Positive and negative control isolates will be run concurrently with each test. Also, an uninoculated slant must be incubated to indicate that there is no change in the phenol red pH indicator system due to atmospheric gases.

   a. Positive - *T. mentagrophytes*
   b. Negative - *T. rubrum*
   c. Uninoculated control

L. Exoantigen Test

1. The exoantigen test is a specific immunodiffusion (ID) test developed to provide rapid information as to the immuno-identity of an unknown isolate. This is done by reacting a concentrate of the organism's soluable antigens against paired positive control reagents for each of the systemic fungi. The test consists of a six-day or older slant of the unknown organism which is extracted overnight in a merthiolate solution and then concentrated 25-50X. The concentrate is placed in two of the peripheral wells of a hexagonal ID matrix, a concentrate derived from one of the deep fungi (control antigen) is placed at the top and bottom of the ID pattern, and both unknown and control are reacted against an antiserum directed toward the control antigen. The test is read at 24 hours and a line(s) of identity with the positive control is (are) considered a positive identification.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

2. The advantages of this procedure are several-fold. Final identification can occur within 24 hours after the fungus on the slant has developed characteristic morphology (i.e., 6-10 days) without having to wait an additional two to six weeks for conversion to the tissue phase. Sterile Mycelia, i.e., those organisms which do not form recognizable conidial structures, may be extracted and their relationship to the deep fungi determined. This is particularly important since primary isolates of *Histoplasma*, *Blastomyces*, and *Coccidioides* sometimes do not exhibit their characteristic microscopic morphology. Finally, any laboratory which has a biological safety cabinet and access to the control reagents may do the test, reducing the necessity of delaying the final report until a reference laboratory confirms the identification.

3. Procedure

   a. Preparation of ID Agar (1% phenolized agar medium, pH 6.3-6.4)

      Sodium chloride (NaC1)
      0.9 gm Sodium citrate ($Na_3C_6H_5O_7.2\ H_2O$)
      0.4 gm Phenol (aqueous 90% w/w)
      0.25 ml Glycine
      7.5 gm Purified agar (Difco)
      1.0 gm Distilled water, qs to 100 ml

   b. Add 50.0 ml of distilled water to a 125 ml screw cap Erlenmeyer flask.
   c. Add 0.9 gm of sodium chloride and 0.4 gm of sodium citrate to the flask. Completely dissolve both salts.
   d. Add 0.25 ml of liquid phenol (90% w/w) and 7.5 gm of glycine to the flask. Mix thoroughly.
   e. Add 1.0 gm of purified agar (Difco) and make up to 100 ml with distilled water.
   f. Autoclave for 10 minutes at 120ºC. (Loosen cap on flask before autoclaving). The final pH of the agar must be 6.3-6.4. Adjust pH if necessary.

4. Preparation of Immunodiffusion Plates

   a. The micro ID agar plate is prepared by pipetting 6.5 ml of melted glycine-phenol agar (60-65°C) into a 100 X 15 mm plastic Petri dish. This forms the base layer.
   b. The base layer is allowed to set for at least 30 minutes.
   c. Three and one half ml of hot glycine-phenol agar is pipetted onto one side of the solidified base layer of the agar. Immediately lower and press a 17-7 well microimmunodiffusion template into the hot agar in a manner to avoid entrapment of air bubbles. This secures the template to the basal agar layer.
   d. The plate is allowed to sit for at least 30 minutes before us.
   e. After agar has gelled, the extra agar is cleared off of the template using a 1 mm spatula. Placing the plates in a refrigerator for an additional 5-10 minutes before cleaning facilitaties the cleaning of the agar from the wells.

      NOTE: These plates can be stored up to four weeks if kept in a moist chamber at 4°C.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

5. Extraction of Exoantigens

   a. A mature culture with at least 15 X 30 mm of growth on a Sabouraud dextrose agar slant (in 20 X 150 mm tube with 10 ml agar) is covered with 8-10 ml of a 1:5000 aqueous solution of Thimerosal using a Pasteur pipette.
   b. The solution is permitted to extract with mycelial growth for at least 24 hours at room temperature.
   c. Five milliliters of each cellular extract is then transferred to an Amicon Minicon macrosolute B-15 concentrator with a Pasteur pipette. Place one drop of remaining extract on any media for sterility test.
   d. A wet mount is prepared from the killed cultures.
   e. Extracts derived from cultures demonstrating arthroconidia and suspected of being *C. immitis* are concentrated 25X.
   f. Extracts from cultures demonstrating tuberculated macroconidia and suspected of being *H. capsulatum*, as well as those exhibiting pyriform microconidia consistent with the mould phase of *Blastomyces dermatitidis*, are concentrated 50X.
   g. Cultures without characteristic conidia are tested for antigens against all three pathogens.

6. Performance of the ID Test for the Identification of Fungal Cultures

   a. Control antiserum is placed in the center well of the pattern to be used. Before proceeding to the next step, the serum must be allowed to diffuse for one hour at room temperature.
   b. The control antigens are placed in the upper and lower wells and duplicate unknown supernatant antigens are placed in the lateral wells on the same slide.

      NOTE: Extracts of the same culture should be set up on the same side of the control antigen since an extract containing a strong antigen may produce such an intense reaction so as to make a negative extract in an adjacent well appear weakly positive.

   c. The charged immunodiffusion plates are placed in a moist chamber at room temperature for 24 hours.
   d. After 24 hours the ID templates are carefully removed. The agar surface is washed and then covered with distilled water, after which the plates are read. If the reactions are not clear the agar surface may be washed free of excess agar, covered with distilled water, and re-examined again.

7. Interpretation

   A fungus is identified as *Histoplasma capsulatum* when its concentrated extract contains antigens identical (lines of identity) to H or H and M precipitinogens; as *Blastomyces dermatitidis* when lines of identity occur between the *Blastomyces* control reagents (specifically the A band) and the unknown. An isolate is identified as C. *immitis* when its concentrate produces lines of identity with positive reagents to the tube precipitinogen (TP), heat-labile precipitinogen (HL), or the heat-labile F (CF) precipitinogen.

8. Quality Control - See 2.11.1.(4), Performance of the ID Test, steps 1 and 2.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

M. Slide Culture

1. Principle: Accurate identification of filamentous moulds is based on microscopic examination of the sporulating parts of a colony. While tease preparations are useful, they have two main disadvantages: 1) If too much growth is removed, or not teased apart well, or if the material is taken from a non-sporulating area, it may be difficult to discern sporulating structures. 2) Spores and conidia are often disrupted during preparation of the mount. The slide culture system consists of a mini incubation chamber designed to produce optimum conditions for sporulation. It allows for examination of the colony in various stages of development, and improves the chances of observing the natural configuration of the spores and conidia on the sporulating structures.

2. Specimen: Living cultures are necessary for preparing slide cultures.

3. Quality Assurance: The presence of growth verifies the slide culture has been set-up appropriately.

4. Procedure

   a. Prepare sterile slide culture dishes.

      (1) Line the bottom of a glass dish with filter paper of the same diameter.
      (2) Lay a V-shaped piece of bent glass tubing on the filter paper, top with a clean microscope slide. Place a 22 x 22 mm cover slip (or a 24 x 40 mm for fast-growing fungi) on the filter paper.
      (3) Replace the lid and autoclave the unit for 25 min at 121°C.
      (4) Several slide culture dishes can be assembled, presterilized and stored in a metal box. Because they hold the moisture better and allow more room to manoeuver, glass petri dishes are preferred.

   b. Select a medium known to induce sporulation for the fungus under study. Media like Pablum Cereal Agar and Potato Dextrose Agar, etc. are recommended because they stimulate sporulation in Hyphomycetes. Pablum Cereal Agar has a soft consistency that allows good adherence to the glass surfaces. When pressed gently, the agar block flattens slightly and air bubbles are squeezed out. More solid media such as Potato Dextrose Agar must be cut thinly and smoothly into blocks. Excess pressure in the agar block will cause it to crack, producing a fragmented preparation. Refer to Media section for recipes.
   c. Pour a thin layer of agar into a sterile plastic petri dish and cut a block of agar 2 mm square and approximately 1 mm thick. Place the block of agar on the microscope slide.
   d. Using aseptic technique throughout, remove material from a sporulating part of the colony with a firm needle and inoculate 2-3 areas of the block. Place the coverslip on top of the block using forceps, and press gently.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

e. Add 1-2 ml sterile water to the filter paper, replace the petri dish cover, incubate and observe for sporulation at regular intervals by examining the preparation under a dissecting or compound microscope. Keep the preparation moist by adding additional water when needed. The proximity of the glass and the agar plus the humid atmosphere in the dish encourages hyphal development along the glass. Avoid too much water, which allows for condensation to form on the petri dish lid, which then can drip onto the slide. Mount the fungus when it is sporulating, but before the fungus has overgrown the coverslip, which, usually is between 7-21 days.

f. Permanent mounts may be prepared by using a mounting medium such as glycerine jelly or polyvinyl alcohol. Mounts prepared in these media should be heated gently on a warming tray to seat the coverslips, remove the air bubbles and spread the mounting medium to the edge of the slide. Semi-permanent mounts may be prepared in lacto-fuchsin and the cover slip ringed with nail polish. Be careful to remove air bubbles.

g. Systemic pathogens should be killed by exposure to formaldehyde before mounting. **This should not be performed without prior approval of the director or supervisor.**

h. To mount the coverslip, remove it from the agar block using forceps. Flood the area of growth with wetting agent, drain and mount, fungus-side-down, onto a drop of mounting medium placed on a clean microscope slide. When mounting the coverslip, align it carefully upon the mounting medium in order to prevent disrupting the preparation.

i. To mount the slide, hold it with forceps, lift off the agar block with an inoculating needle, flood the area of growth with wetting agent and drain. Wipe the reverse, top and bottom of the slide with a tissue soaked in disinfectant, leaving the growing area undisturbed. Place a drop of mounting medium in the center of the growth and apply a coverslip.

j. Label the slides using a permanent marker, with the identifying name or number and other pertinent details such as the medium used, age and the fact that it is a slide culture.

5. Results: Sporulation of microfungi will be evident with modes of conidiogenesis being clearly visible for study.

6. Reference:

McGinnis, MR: Laboratory Handbook of Medical Mycology. New York, Academic Press, 1980.

N. *Blastomyces dermatitidis* Culture Identification Kit

1. Principle: The ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST is a rapid DNA probe test which utilizes the technique of nucleic acid hybridization for the identification of *Blastomyces dermatitidis* isolated from culture. Nucleic acid hybridization tests are based on the ability of complementary nucleic acid strands to specifically align and associate to form stable double-stranded complexes (3). The ACCUPROBE SYSTEM uses a chemiluminescent labeled, single-stranded DNA probe, that is complementary to the ribosomal RNA of the target organism. After the ribosomal RNA is released from the organism, the labeled DNA probe combines with the target organism's ribosomal RNA to form a stable DNA:RNA hybrid. The

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

Selection Reagent allows for the differentiation of non-hybridized and hybridized probe. The labeled DNA:RNA hybrids are measured in the luminometer. A positive result is a luminometer reading equal to or greater than the cut-off. A value less than this cut-off is a negative result.

2. Reagents: Reagents for the ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST are provided in three separate reagent kits:

   a. ACCUPROBE BLASTOMYCES DERMATITIDIS PROBE KIT

      (1) Probe Reagent (10 x 2 tubes); *Blastomyces dermatitidis*
      (2) Lysing Reagent (1 x 20 tubes); Glass beads and buffer

   b. ACCUPROBE CULTURE IDENTIFICATION REAGENT KIT

      (1) Reagent 1 (Lysis Reagent) 1 x 10 ml; buffered solution containing 0.04% sodium azide
      (2) Reagent 2 (Hybridization Buffer) 1 x 10 ml; buffered solution
      (3) Reagent 3 (Selection Reagent) 1 x 60 ml; buffered solution

   c. GEN-PROBE DETECTION REAGENT KIT

      (1) Detection Reagent I 1 x 240 ml; 0.1% hydrogen peroxide in 0.001 N nitric acid
      (2) Detection Reagent II 1 x 240 ml; 1 N sodium hydroxide

3. Warnings and precautions:

   a. For i*n vitro* diagnostic use.
   b. Use universal precautions when performing this assay (1).
   c. Use only for the determination of *B. dermatitidis* isolated from culture.
   d. Use only supplied or specified disposable laboratory ware.
   e. Culture handling and all procedural steps through the heat inactivation step should be performed in a Class II Biological Safety Cabinet.
   f. Reagents in this kit contain sodium azide which may react with lead or copper plumbing to form potentially explosive metal azides. Upon disposal of these reagents, always dilute the material with a large volume of water to prevent azide buildup in the plumbing.
   g. Avoid contact of Detection Reagents I and II with skin and mucous membranes. Wash with water if these reagents come into contact with skin. If spills of these reagents occur, dilute with water before wiping dry.

4. Storage and handling requirements:

   a. Probe Reagent Tubes must be stored in the foil pouches at 2° - 8°C. The Probe Reagent Tubes are stable in the unopened pouches until the expiration date indicated. Once opened, the pouch should be resealed and the tubes should be used within two months and prior to the expiration date.
   b. Other reagents used in the ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST may be stored between 2° - 25°C and are stable until the expiration date indicated.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

c. DO NOT FREEZE THE REAGENTS.

5. Sample collection and preparation: The ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST is designed to determine the identity of *B. dermatitidis* isolated from culture. Colonies may be tested as soon as growth is visible. **Yeast colonies should be no more than one week old from the time growth is first observed. Filamentous colonies should be no more than one month old from the time growth is first observed. Sporulation is not necessary.**

   a. **Solid Media Method.** Growth from appropriate solid media such as Sabouraud Dextrose, Brain Heart Infusion, Mycobiotic (Mycosel), Inhibitory Mold Agar, Cottonseed Agar, Corn Meal Agar, Yeast Nitrogen Base Agar or 5% Sheep Blood Agar suggestive of *B. dermatitidis* may be tested.

      (1) Growth can be removed with a 1 µL disposable plastic loop, a wire loop, a disposable plastic needle, or an applicator stick. Swabs should not be used due to the small volume of liquid in which the cells are subsequently resuspended.
      (2) Avoid taking large amounts of the solid media with the cells.
      (3) A 1-2 mm^2 size sample of yeast or filamentous growth is recommended for the test.
      (4) The operator may elect to inoculate another culture plate at this time to confirm the purity of the isolate.

   b. **Broth Culture Method.** Growth in Brain Heart Infusion broth with turbidity equivalent to or greater than a McFarland 1 Nephelometer Standard may be tested with the ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST. Pipette a 100 µL sample from the well-mixed broth suspension into the Lysing Reagent Tube as described below.

6. Materials provided: The ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST

Cat. No. 2890	**20 Tests**
Probe Reagent	10 x 2 tubes
Lysing Reagent	1 x 20 tubes

7. Materials required but not provided:

   a. 1 µL plastic sterile inoculating loops, wire loops, plastic needles, or applicator sticks for selecting colonies
   b. Control culture strains
   c. Water bath or heating block (60° ± 1°C)
   d. Water bath or heating block (95° ± 5°C)
   e. Micropipettes (100 µL, 300 µL)
   f. Re-pipettor (100 µL, 300 µL)
   g. Vortex mixer
   h. AVAILABLE FROM GEN-PROBE:

      (1) GEN-PROBE Luminometer: LEADER or ACCULDR (formerly PAL)
      (2) GEN-PROBE Sonicator or equivalent

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

(3) ACCUPROBE CULTURE IDENTIFICATION REAGENT KIT (Cat.No.2800)
(4) GEN-PROBE DETECTION REAGENT KIT (Cat.No.1791)
(5) GEN-PROBE Heating Block (Cat.No.2775)
(6) GEN-PROBE Sonicator Rack (Cat.No.1357)

8. Test procedure:

a. Equipment Preparation

(1) For optimal transfer of sonic energy, water must be thoroughly degassed according to the following procedure:

(a) Add enough water to fill the sonicator bath to within 1/2 inch of the top of the tank.
(b) Run the sonicator for 15 minutes to thoroughly degas the water. Sonicator should be used within a Biological Safety Cabinet.

(2) Adjust one heating block or water bath to 60° ± 1°C and another heating block or water bath to 95° ± 5°C.
(3) Prepare the Gen-Probe luminometer for operation. Make sure there is sufficient volume of Detection Reagents I and II to complete the tests.

b. Controls: Positive and negative control strains should be tested routinely with each batch of test run. A culture of *B. dermatitidis* (UTMB 3538) may be used as the positive control, while a culture of *H. capsulatum* (UTMB 3537) may be used as the negative control.
c. Sample preparation:

(1) Label a sufficient number of Lysing Reagent Tubes to test the culture isolates and/or controls. Remove and retain the caps.
(2) Pipette 100 µL of Reagent 1 (Lysis Reagent) and 100 µL of Reagent 2 (Hybridization Buffer) into all Lysing Reagent Tubes. **If broth cultures are to be tested, do not add Reagent 1 to the Lysing Reagent Tubes.**
(3) Transfer the sample from the solid media or 100 µL of a well mixed broth culture into the labeled Lysing Reagent Tubes as described in the SAMPLE COLLECTION AND PREPARATION Section. Twirl the loop, needle or stick in the Reagent 1 and Reagent 2 diluent mixture to remove the cells if testing growth from solid media.
(4) Recap the Lysing Reagent Tubes and briefly VORTEX .

d. Sample Lysis

(1) Push the Lysing Reagent Tubes through the Sonicator Rack so that the reaction mixture in the bottom of the tube is submerged but the caps are above the water. Place Sonicator Rack on water bath sonicator. DO NOT ALLOW THE TUBES TO TOUCH THE BOTTOM OR SIDES OF THE SONICATOR.
(2) Sonicate for 15 minutes.
(3) Place the Lysing Reagent Tubes, containing the sonicated organisms in a heating block or water bath for 10 minutes at 95° ± 5°C.
(4) Carefully remove the Lysing Reagent Tubes from the heating block or water bath.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

e. Hybridization

(1) Open the foil pouch by cutting evenly across the top of the pouch. Remove enough Probe Reagent Tubes to test the culture isolates and/or controls. Reseal the pouch by folding the opened edge over several times and securing with adhesive tape or a clip. **Leave the desiccant pillow in the pouch.**
(2) Label a sufficient number of Probe Reagent Tubes to test the culture isolates and/or controls. Remove and retain caps.
(3) Pipette 100 µL of the lysed specimens from the Lysing Reagent Tubes into the corresponding Probe Reagent Tubes.
(4) Recap the Probe Reagent Tubes and incubate for 15 minutes at 60° ± 1°C in a water bath or heating block.

f. Selection

(1) Remove the Probe Reagent Tubes from the water bath. Remove and retain the caps. Pipette 300 µL of Reagent 3 (Selection Reagent) into each tube. If a heating block is used, Reagent 3 may be pipetted directly into the tubes. Recap the tubes and VORTEX them to mix completely.
(2) Incubate the Probe Reagent Tubes for 5 minutes at 60° ± 1°C in a water bath or heating block.
(3) Remove the Probe Reagent Tubes from the water bath or heating block and leave them at room temperature for at least 5 minutes. Remove and discard the caps. **Read the results in the luminometer within 1 hour after removing from water bath or heating block.**

g. Detection

(1) Select the appropriate protocol from the menu of the luminometer software.
(2) Using a damp tissue or paper towel, wipe each tube to ensure that no residue is present on the outside of the tube and insert the tubes into the luminometer according to the instrument directions.
(3) When the analysis is complete, remove the tube(s) from the luminometer.

9. Procedural notes:

a. REAGENTS: Reagent 2 (Hybridization Buffer) may precipitate. Warming and mixing the solution at 35° - 60° will dissolve the precipitate.
b. TEMPERATURE: The Hybridization and Selection reactions are temperature dependent. Therefore, it is imperative that the water bath or heating block is maintained within the specified temperature range.
c. TIME: The Hybridization and Selection reactions are time dependent. Hybridize at least 15 minutes but no more than 20 minutes. Incubate the Probe Reagent Tubes during the SELECTION Step for at least 5 minutes but no more than 6 minutes.
d. WATER BATH: The level of water in the water bath should be maintained to ensure that the Lysing Reagent Tubes are submerged up to, but not above, the level of the sealing ring. It should also be ensured that the entire liquid reaction volume in the Probe Reagent Tubes is submerged.
e. VORTEXING: It is critical to have a homogeneous mixture during the SAMPLE PREPARATION and SELECTION Steps, specifically after the addition of cells to Reagents 1 and 2 after the addition of Reagent 3.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

f. TROUBLE-SHOOTING

(1) Elevated negative control values (*H. capsulatum*) greater than 20,000 RLU (Relative Light Units) in the LEADER or 600 PLU (Photometric Light Units) in the ACCULDR (formerly PAL) can be caused by insufficient mixing after adding Reagent 3 (Selection Reagent) or by testing mixed cultures. Because mixed cultures can occur, a portion of the growth may be streaked onto the appropriate agar medium and incubated to check for multiple colony types.

(2) Low positive control valves (*B. dermatitidis*) less than 50,000 RLU in the LEADER or 1,500 PLU in the ACCULDR (formerly PAL) can be caused by insufficient cell numbers, improper sonication or by testing mixed or aged cultures. Because mixed cultures can occur, a portion of the growth may be streaked onto the appropriate agar medium and incubated to check for multiple colony types.

10. Results:

a. Interpretation of Results

The results of the ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST are based on the following cut-off values. Samples producing signals greater than or equal to these cut-off values are considered positive. Signals less than these cut-off values are considered negative. Results in repeat ranges should be repeated.

	ACCULDR™ (formerly PAL)	**LEADER™**
Cut-off value	1,500 PLU	50,000 RLU
Repeat range	1,200-1,499 PLU	40,000-49,999 RLU

b. Quality Control and Acceptability of Results

Negative control and positive control (e.g., *H. capsulatum*, ATCC #38904) should satisfy the following values:

	ACCULDR™ (formerly PAL)	**LEADER™**
Negative control	< 600 PLU	< 20,000 RLU
Positive control	> 1,500 PLU	> 50,000 RLU

11. Limitations:

This method has been tested using fresh growth from solid media and from broth cultures listed in the SAMPLE COLLECTION AND PREPARATION Section. The efficacy of this test has not been demonstrated on direct clinical specimens (e.g., respiratory specimens or CSF). Results from the ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST should be interpreted in conjunction with other laboratory and clinical data available to the clinician.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

12. Expected values:

The ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST was compared to standard culture, morphological and biochemical identification methods at two sites using a total of 396 isolates. 108 were *B. dermatitidis* isolates (11 isolates were tested in both the yeast and the mycelial phases), 1 *P. brasiliensis* isolate, and 287 isolates of 68 species from 76 genera, representing a phylogenetic cross-section of organisms. Included among these were 103 isolates of the following dimorphic fungi: 7 *Coccidioides immitis*; 94 *Histoplasma capsulatum*; 2 *Sporothrix schenckii*. Standard culture identification is dependent on microsopic and macroscopic colony morphology, sporulation characteristics, filamentous to yeast phase conversions, and in some cases, exoantigen tests. Isolates were either categorized as positive ($\geq$ 50,000 RLU) or negative ($<$ 50,000 RLU). The range of observations for negative isolates was 963 RLU to 32,071 RLU and 66,502 RLU to 644,940 RLU for positive isolates. A comparison of the ACCUPROBE BASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST and standard culture identification methods is shown below.

ACCUPROBE/CULTURE IDENTIFICATION

ACCUPROBE Culture	Pos Pos	Pos Neg	Neg Pos	Neg Neg	Sensitivity/ Specificity	Percent Agreement
Site 1	53	1	0	77	100%/98.7%	99.2%
Site 2	53	0	2	210	96.4%/100%	99.2%
Total	**106**	**1**	**2**	**287**	**98.1%/99.7%**	**99.2%**

The one positive result at Site 1 was a *P. barsiliensis* isolate. As discussed in the LIMITATION section, the ACCUPROBE BASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST does not differentiate between *B. dermatitidis* and *P. brasiliensis* isolates since both have been shown to produce positive results. The sensitivity, specificity, and percent agreement, for Site 1, are therefore 100%. The two isolates initially producing a negative result, at Site 2, produced positive results upon retesting with the ACCUPROBE BASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST. The overall sensitivity, specificity and percent agreement, upon retesting are therefore 100%.

13. Performance characteristics:

a. Within-Run Precision

The within-run precision of the ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST was calculated by assaying two concentrations of ribosomal RNA isolated from *B. dermatitidis* using 10 replicates in a single assay.

Sample	**A**	**B**
Number of Replicates	10	10
Mean Response	57,602	135,061
Standard Deviation	5,929	3,698
Coefficient of Variation	10.3%	2.7%

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

b. Between-Run Precision

The between-run precision was calculated by assaying the same two concentrations of *B. dermatitidis* ribosomal RNA using single determinations in 15 consecutive runs.

Sample	**A**	**B**
Number of Replicates	10	10
Mean Response	59,160	180,140
Standard Deviation	6,656	8.537
Coefficient of Variation	11.3%	4.7%

c. Specificity

A total of 102 ATCC reference isolates were evaluated using the ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST. These isolates represented a total of 71 species from 46 genera. Four isolates of *B. dermatitidis*, 4 isolates of *P. brasiliensis*, 6 isolates of *H. capsulatum*, and 1 isolate of *S. schenckii* were tested in the filamentous phase. Of these, a total of 12 isolates were also tested in the yeast phase. Eighty-seven isolates of 42 other genera representing a phylogenetic cross-section of organisms were evaluated using the ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICA-TION TEST. All non-target isolates representing a phyogenetic cross-section of organisms produced negative results using this kit. The ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST does not differentiate between *P. brasilensis* and *B. dermatitidis* isolates. All *P. brasiliensis* isolates tested in both the filamentous and yeast phases produced positive results. All *B. dermatitidis* isolates tested in both the filamentous and yeast phases produced positive results using the ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST. The representative phylogenetic cross-section isolates did not react in this test.

d. Recovery

B. dermatitidis ribosomal RNA at concentrations ranging from 0.04 µg to 1.6 µg per test were assayed alone in the presence of 0.0045 µg (equivalent to 10^5 cells) to 0.45 µg (equivalent to 10^7 cells) ribosomal RNA of either *H. capsulatum* or *Candida albicans*. There was no interference with the *B. dermatitidis* signal observed and the other organisms present did not react using the ACCUPROBE BLASTOMYCES DERMATITIDIS CULTURE IDENTIFICATION TEST.

14. References:

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II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

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O. *Coccidioides immitis* Culture Identification Kit

1. Principle: The ACCUPROBE COCCIDIOIDES IMMITIS CULTURE IDENTIFICATION TEST is a rapid DNA probe test which utilizes the technique of nucleic acid hybridization for the identification of *Coccidioides immitis* isolated from culture. Nucleic acid hybridization tests are based on the ability of complementary nucleic acid strands to specifically align and associate to form stable double-stranded complexes (3). The ACCUPROBE SYSTEM uses a chemiluminescent labeled, single-stranded DNA probe, that is complementary to the ribosomal RNA of the target organism. After the ribosomal RNA is released from the organism, the labeled DNA probe combines with the target organism's ribosomal RNA to form a stable DNA:RNA hybrid. The Selection Reagent allows for the differentiation of non-hybridized and hybridized probe. The labeled DNA:RNA hybrids are measured in the luminometer. A positive result is a luminometer reading equal to or greater than the cut-off. A value less than this cut-off is a negative result.

2. Reagents:

   Reagents for the Accuprobe Coccidioides Immitis Culture Identification Test are provided in three separate reagent kits:

   a. ACCUPROBE COCCIDIOIDES IMMITIS PROBE KIT

      (1) Probe Reagent (10 x 2 tubes); *Coccidioides immitis*
      (2) Lysing Reagent (1 x 20 tubes); Glass beads and buffer

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

b. ACCUPROBE CULTURE IDENTIFICATION REAGENT KIT

(1) Reagent 1 (Lysis Reagent) 1 x 10 ml; buffered solution containing 0.04% sodium azide
(2) Reagent 2 (Hybridization Buffer) 1 x 10 ml; buffered solution
(3) Reagent 3 (Selection Reagent) 1 x 60 ml; buffered solution

c. GEN-PROBE DETECTION REAGENT KIT

(1) Detection Reagent I 1 x 240 ml; 0.1% hydrogen peroxide in 0.001 N nitric acid
(2) Detection Reagent II 1 x 240 ml; 1 N sodium hydroxide

3. Warnings and precautions:

a. For i*n vitro* diagnostic use.
b. Use universal precautions when performing this assay (2). Coccidioides immitis arthroconidia represent a major biohazard to laboratory personnel. Biosafety level 3 is recommended for all activities with mold cultures and adherence to appropriate precautions should be rigoursly follwed (3). Culture samples (e.g., pipetted, vortexed, etc.) and all procedural steps through the heat inactivation stop should be performed in a Class II or III Biological Safety Cabinet or a Bacteriological Glove Box.

(1) Arthrospore aerosois may be prevented by wetting growth in the mycelium phase with one or two drops of sterile distilled water.
(2) Appropriate autoclaving procedures for all contaminated materials should be strictly followed.

c. Use only for the determination of *C. immitis* isolated from culture.
d. Use only supplied or specified disposable laboratory ware.
e. Use routine laboratory precautions. Wash hands thoroughly after handling specimens and kit reagents.
f. Reagents in this kit contain sodium azide which may react with lead or copper plumbing to form potentially explosive metal azides. Upon disposal of these reagents, always dilute the material with a large volume of water to prevent azide buildup in the plumbing.
g. Avoid contact of Detection Reagents I and II with skin and mucous membranes. Wash with water if these reagents come into contact with skin. If spills of these reagents occur, dilute with water before wiping dry.

4. Storage and handling requirements:

a. Probe Reagent Tubes must be stored in the foil pouches at 2° - 8°C. The Probe Reagent Tubes are stable in the unopened pouches until the expiration date indicated. Once opened, the pouch should be resealed and the tubes should be used within two months and prior to the expiration date.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

b. Other reagents used in the ACCUPROBE COCCIDIOIDES IMMITIS CULTURE IDENTIFICATION TEST may be stored between 2° - 25°C and are stable until the expiration date indicated.
c. DO NOT FREEZE THE REAGENTS.

5. Sample collection and preparation:

The ACCUPROBE COCCIDIOIDES IMMITIS CULTURE IDENTIFICATION TEST is designed to determine the identity of *C. immitis* isolated from culture. Colonies may be tested as soon as growth is visible. **Yeast colonies should be no more than one week old from the time growth is first observed. Filamentous colonies should be no more than one month old from the time growth is first observed. Sporulation is not necessary.**

a. **Solid Media Method.** Growth from appropriate solid media such as Sabouraud Dextrose, Brain Heart Infusion, Mycobiotic (Mycosel), Inhibitory Mold Agar, Cottonseed Agar, Corn Meal Agar, Yeast Nitrogen Base Agar or 5% Sheep Blood Agar suggestive of *C. immitis* may be tested.

(1) Growth can be removed with a 1 µL disposable plastic loop, a wire loop, a disposable plastic needle, or an applicator stick. Swabs should not be used due to the small volume of liquid in which the cells are subsequently resuspended.
(2) Avoid taking large amounts of the solid media with the cells.
(3) A 1-2 mm^2 size sample of yeast or filamentous growth is recommended for the test.
(4) The operator may elect to inoculate another culture plate at this time to confirm the purity of the isolate.

b. Broth Culture Method. Growth in Brain Heart Infusion broth with turbidity equivalent to or greater than a McFarland 1 Nephelometer Standard may be tested with the ACCUPROBE COCCIDIOIDES IMMITIS CULTURE IDENTIFICATION TEST. Pipette a 100 µL sample from the well-mixed broth suspension into the Lysing Reagent Tube as described below.

6. Materials provided:

The ACCUPROBE COCCIDIOIDES IMMITIS CULTURE IDENTIFICATION TEST

Cat. No. 2895	**20 Tests**
Probe Reagent	10 x 2 tubes
Lysing Reagent	1 x 20 tubes

7. Materials required but not provided:

a. 1 µL plastic sterile inoculating loops, wire loops, plastic needles, or applicator sticks for selecting colonies
b. Control culture strains
c. Water bath or heating block (60° ± 1°C)
d. Water bath or heating block (95° ± 5°C)
e. Micropipettes (100 µL, 300 µL)

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

f. Re-pipettor (100 µL, 300 µL)
g. Vortex mixer
h. AVAILABLE FROM GEN-PROBE:

(1) GEN-PROBE Luminometer: LEADER or ACCULDR (formerly PAL)
(2) GEN-PROBE Sonicator or equivalent
(3) ACCUPROBE CULTURE IDENTIFICATION REAGENT KIT (Cat.No.2800)
(4) GEN-PROBE DETECTION REAGENT KIT (Cat.No.1791)
(5) GEN-PROBE Heating Block (Cat.No.2775)
(6) GEN-PROBE Sonicator Rack (Cat.No.1357)

8. Test procedure:

a. Equipment Preparation

(1) For optimal transfer of sonic energy, water must be thoroughly degassed according to the following procedure:

(a) Add enough water to fill the sonicator bath to within 1/2 inch of the top of the tank.
(b) Run the sonicator for 15 minutes to thoroughly degas the water. Sonicator should be used within a Biological Safety Cabinet.

(2) Adjust one heating block or water bath to 60° ± 1°C and another heating block or water bath to 95° ± 5°C.
(3) Prepare the Gen-Probe luminometer for operation. Make sure there is sufficient volume of Detection Reagents I and II to complete the tests.

b. Controls: Positive and negative control strains should be tested routinely with each batch of test run. A culture of *C. immitis* (UTMB 3536) may be used as the positive control, while a culture of *Blastomyces dermatitidis* (UTMB 3538) may be used as the negative control.
c. Sample Preparation

(1) Label a sufficient number of Lysing Reagent Tubes to test the culture isolates and/or controls. Remove and retain the caps.
(2) Pipette 100 µL of Reagent 1 (Lysis Reagent) and 100 µL of Reagent 2 (Hybridization Buffer) into all Lysing Reagent Tubes. **If broth cultures are to be tested, do not add Reagent 1 to the Lysing Reagent Tubes.**
(3) Transfer the sample from the solid media or 100 µL of a well mixed broth culture into the labeled Lysing Reagent Tubes as described in the SAMPLE COLLECTION AND PREPARATION Section. Twirl the loop, needle or stick in the Reagent 1 and Reagent 2 diluent mixture to remove the cells if testing growth from solid media.
(4) Recap the Lysing Reagent Tubes and briefly VORTEX .

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

d. Sample Lysis

(1) Push the Lysing Reagent Tubes through the Sonicator Rack so that the reaction mixture in the bottom of the tube is submerged but the caps are above the water. Place Sonicator Rack on water bath sonicator. DO NOT ALLOW THE TUBES TO TOUCH THE BOTTOM OR SIDES OF THE SONICATOR.
(2) Sonicate for 15 minutes.
(3) Place the Lysing Reagent Tubes, containing the sonicated organisms in a heating block or water bath for 10 minutes at 95° ± 5°C.
(4) Carefully remove the Lysing Reagent Tubes from the heating block or water bath.

e. Hybridization

(1) Open the foil pouch by cutting evenly across the top of the pouch. Remove enough Probe Reagent Tubes to test the culture isolates and/or controls. Reseal the pouch by folding the opened edge over several times and securing with adhesive tape or a clip. **Leave the desiccant pillow in the pouch.**
(2) Label a sufficient number of Probe Reagent Tubes to test the culture isolates and/or controls. Remove and retain caps.
(3) Pipette 100 µL of the lysed specimens from the Lysing Reagent Tubes into the corresponding Probe Reagent Tubes.
(4) Recap the Probe Reagent Tubes and incubate for 15 minutes at 60° ± 1°C in a water bath or heating block.

f. Selection

(1) Remove the Probe Reagent Tubes from the water bath. Remove and retain the caps. Pipette 300 µL of Reagent 3 (Selection Reagent) into each tube. If a heating block is used, Reagent 3 may be pipetted directly into the tubes. Recap the tubes and VORTEX them to mix completely.
(2) Incubate the Probe Reagent Tubes for 5 minutes at 60° ± 1°C in a water bath or heating block.
(3) Remove the Probe Reagent Tubes from the water bath or heating block and leave them at room temperature for at least 5 minutes. Remove and discard the caps. **Read the results in the luminometer within 1 hour after removing from water bath or heating block.**

g. Detection

(1) Select the appropriate protocol from the menu of the luminometer software.
(2) Using a damp tissue or paper towel, wipe each tube to ensure that no residue is present on the outside of the tube and insert the tubes into the luminometer according to the instrument directions.
(3) When the analysis is complete, remove the tube(s) from the luminometer.

9. Procedural notes:

a. REAGENTS: Reagent 2 (Hybridization Buffer) may precipitate. Warming and mixing the solution at 35° - 60° will dissolve the precipitate.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

b. TEMPERATURE: The Hybridization and Selection reactions are temperature dependent. Therefore, it is imperative that the water bath or heating block is maintained within the specified temperature range.
c. The Hybridization and Selection reactions are time dependent. Hybridize at least 15 minutes but no more than 20 minutes. Incubate the Probe Reagent Tubes during the SELECTION Step for at least 5 minutes but no more than 6 minutes.
d. WATER BATH: The level of water in the water bath should be maintained to ensure that the Lysing Reagent Tubes are submerged up to, but not above, the level of the sealing ring. It should also be ensured that the entire liquid reaction volume in the Probe Reagent Tubes is submerged.
e. VORTEXING: It is critical to have a homogeneous mixture during the SAMPLE PREPARATION and SELECTION Steps, specifically after the addition of cells to Reagents 1 and 2 after the addition of Reagent 3.
f. TROUBLE-SHOOTING

(1) Elevated negative control values (*B. dermatitidis*) greater than 20,000 RLU (Relative Light Units) in the LEADER or 600 PLU (Photometric Light Units) in the ACCULDR (formerly PAL) can be caused by insufficient mixing after adding Reagent 3 (Selection Reagent) or by testing mixed cultures. Because mixed cultures can occur, a portion of the growth may be streaked onto the appropriate agar medium and incubated to check for multiple colony types.
(2) Low positive control valves (*C. immitis*) less than 50,000 RLU in the LEADER or 1,500 PLU in the ACCULDR (formerly PAL) can be caused by insufficient cell numbers, improper sonication or by testing mixed or aged cultures. Because mixed cultures can occur, a portion of the growth may be streaked onto the appropriate agar medium and incubated to check for multiple colony types.

10. Results:

a. Interpretation of Results: The results of the ACCUPROBE COCCIDIOIDES IMMITIS CULTURE IDENTIFICATION TEST are based on the following cut-off values. Samples producing signals greater than or equal to these cut-off values are considered positive. Signals less than these cut-off values are considered negative. Results in repeat ranges should be repeated.

	ACCULDR™ (formerly PAL)	**LEADER™**
Cut-off value	1,500 PLU	50,000 RLU
Repeat range	1,200-1,499 PLU	40,000-49,999 RLU

b. Quality Control and Acceptability of Results: Negative control (e.g., *B. dermatitidis*) and positive control (e.g., *C. immitis*) should satisfy the following values:

	ACCULDR™ (formerly PAL)	**LEADER™**
Negative control	<600 PLU	<20,000 RLU
Positive control	>1,500 PLU	>50,000 RLU

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

11. Limitations: This method has been tested using fresh growth from solid media and from broth cultures listed in the SAMPLE COLLECTION AND PREPARATION Section. The efficacy of this test has not been demonstrated on direct clinical specimens (e.g., respiratory specimens or CSF). Results from the ACCUPROBE COCCIDIOIDES IMMITIS CULTURE IDENTIFICATION TEST should be interpreted in conjunction with other laboratory and clinical data available to the clinician.

12. Expected values: The ACCUPROBE COCCIDIOIDES IMMITIS CULTURE IDENTIFICATION TEST was compared to standard culture morphological and biochemical identification methods, for the identification of *C. immitis* at 2 sites using a total of 471 isolates. These isolates were comprised of 166 *C. immitis* isolates and 305 isolates representing 82 species from 77 genera. These non-target isolates represented a wide phylogenetic cross-section of organisms. Standard culture identification included selective growth media, biochemical identification methods, microscopic and macroscopic colony morphology, sporulation characteristics and in some cases, exoantigen tests. Isolates were either categorized as posibive ($\geq$ 50,000 RLU) or negative (< 50,000 RLU). The range of observations for negative isoaltes was 346 RLU to 34,301 RLU and 67,641 RLU to 812,451 RLU for positive isolates. A comparison of the ACCUPROBE COCCIDIOIDES IMMITIS CULTURE INDENTIFICATION TEST and standard culture identification methods is shown below.

ACCUPROBE/CULTURE IDENTIFICATION

ACCUPROBE Culture	Pos Pos	Pos Neg	Neg Pos	Neg Neg	Sensitivity/ Specificity	Percent Agreement
Site 1	84	0	0	130	97.7%/100%	99.1%
Site 2	80	0	0	175	100%/100%	100%
Total	**164**	**0**	**2**	**305**	**98.8%/100%**	**99.6%**

13. Performance characteristics:

a. Within-Run Precision: The within-run precision of the ACCUPROBE COCCIDIOIDES IMMITIS CULTURE IDENTIFICATION TEST was calculated by assaying two concentrations of ribosomal RNA isolated from *C. immitis* using 10 replicates in a single assay.

Sample	**A**	**B**
Number of Replicates	10	10
Mean Response	42,027	136,815
Standard Deviation	2,370	7,004
Coefficient of Variation	5.6%	5.1%

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

b. Between-Run Precision: The between-run precision was calculated by assaying the same two concentrations of *C. immitis* ribosomal RNA using single determinations in 15 consecutive runs.

Sample	**A**	**B**
Number of Replicates	10	10
Mean Response	51,104	150,682
Standard Deviation	2,924	12,449
Coefficient of Variation	5.7%	8.3%

c. Specificity: A total of 106 ATCC culture isolates were evaluated using the ACCUPROBE COCCIDIOIDES IMMITIS CULTURE IDENTIFICATION TEST. These isolates represented a total of 71 species from 45 genera. Six isolates of THE TOTAL NUMBER OF ISOLATES TESTED WERE *Coccidioides immitis* isolates. Six isolates of *H. capsulatum*, 4 isolates of *B. dermatitidis*, 3 isolates of *P. brasiliensis* and 1 isolate of *S. schenckii* were tested in the filamentous phase. Twelve of these isolates were also tested in the yeast phase. Eithy-six isolates of 39 other genera representing a phylogenetic cross-section of fungal organisms were evaluated using the ACCUPROBE COCCIDIOIDES IMMITIS CULTURE IDENTIFICATION TEST. Included among the non-target genera representing a phylogenetic cross-section were species of Malbranchea, Uncinocarpus, Myxotrichum, Oidiodendron, Arachniotus, Gymnoascus, Geotrichum, and Trichosporon. All non-target isolates produced negative results using this test in both the filamentous and the yeast phases. All *Coccidioides immitis* isolates tested produced a positive result using the ACCUPROBE COCCIDIOIDES CULTURE IDENTIFICATION TEST.

d. Recovery: *Coccidioides immitis* ribosomal RNA (rRNA) at concentrations ranging from 0.09 µg to 0.27 µg per test was assayed alone and in the presence of *Histoplasma capsulatums* or *Candida albicans* ranging from 0.002 µg (equivalent to 5×10^4 cells) to 0.2 µg (equivalent to 5×10^6 cells). The presence of these non-target rRNA concentrations did not interfere with the positive reaction with the ACCUPROBE COCCIDIOIDES IMMITIS CULTURE IDENTIFICATION TEST.

14. References:

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c. Centers for Disease Control and National Institutes of Health, 1988. Biosafety in microbiological and biomedical laboratories. US Dept of Health and Human Services, HHS Publication No. (CDC) 84-8395. US Government Printing Office, Washington, DC.
d. Kohne, DE, Steigerwalt, AG. Brenner, DG: Nucleic acid probe specific for members of the genus Legionella. In Legionella, Proceedings of the 2nd International Symposium, C. Thornsberry, C, *et al*. (ed) Washington, DC, , American Society for Microbiology, pp, 107-108, 1984.
e. Koneman, EW, Roberts, GD: Practical laboratory mycology, Third Ediction, Baltimore, Williams & Wilkins, pp. 113-115, 1985.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

f. Rippon, JW: Coccidioidomycosis, Medical Mycology, Third Edition, Philadelphia, W. B. Saunders Company, p. 433, 1988.
g. Standard, PG, Kaufman, L: Immunological procedure for the rapid and specific identification of *Coccidioidomycosis immitis* cultures. J. Clin. Microbio. 5:149-153, 1977.
h. Stevens, DA, Coccidioides immitis. In Prinipals and Practice of Infectious Diseases, Mandell, G, Douglas, R, Bennett, J (ed) Third Edition, New York, Churchill Livingston, p. 2008, 1990.
i. Wellch, HA, Coccidioides immits, International Textbook of Medicine, Medical Microbiology, and Infectious Diseases, Philadelphia, W. B. Saunders Company, p. 658, 1981.

P. *Histoplasma capsulatum* Culture Identification Kit:

1. Principle: The ACCUPROBE HISTOPLASMA CAPSULATUM CULTURE IDENTIFICATION TEST is a rapid DNA probe test which utilizes the technique of nucleic acid hybridization for the identification of *Histoplasma capsulatum* isolated from culture. Nucleic acid hybridization tests are based on the ability of complementary nucleic acid strands to specifically align and associate to form stable double-stranded complexes (3). The ACCUPROBE SYSTEM uses a chemiluminescent labeled, single-stranded DNA probe, that is complementary to the ribosomal RNA of the target organism. After the ribosomal RNA is released from the organism, the labeled DNA probe combines with the target organism's ribosomal RNA to form a stable DNA:RNA hybrid. The Selection Reagent allows for the differentiation of non-hybridized and hybridized probe. The labeled DNA:RNA hybrids are measured in the luminometer. A positive result is a luminometer reading equal to or greater than the cut-off. A value less than this cut-off is a negative result.

2. Reagents: Reagents for the Accuprobe Histoplasma Capsulatum Culture Identification Test are provided in three separate reagent kits:

   a. ACCUPROBE HISTOPLASMA CAPSULATUM PROBE KIT

      (1) Probe Reagent (10 x 2 tubes); *Histoplasma capsulatum*
      (2) Lysing Reagent (1 x 20 tubes); Glass beads and buffer

   b. ACCUPROBE CULTURE IDENTIFICATION REAGENT KIT

      (1) Reagent 1 (Lysis Reagent) 1 x 10 ml; buffered solution containing 0.04% sodium azide
      (2) Reagent 2 (Hybridization Buffer) 1 x 10 ml; buffered solution
      (3) Reagent 3 (Selection Reagent) 1 x 60 ml; buffered solution

   c. GEN-PROBE DETECTION REAGENT KIT

      (1) Detection Reagent I 1 x 240 ml; 0.1% hydrogen peroxide in 0.001 N nitric acid
      (2) Detection Reagent II 1 x 240 ml; 1 N sodium hydroxide

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

3. Warnings and precautions:

   a. For i*n vitro* diagnostic use.
   b. Use universal precautions when performing this assay (1).
   c. Use only for the determination of *H. capsulatum* isolated from culture.
   d. Use only supplied or specified disposable laboratory ware.
   e. Culture handling and all procedural steps through the heat inactivation step should be performed in a Class II Biological Safety Cabinet.
   f. Reagents in this kit contain sodium azide which may react with lead or copper plumbing to form potentially explosive metal azides. Upon disposal of these reagents, always dilute the material with a large volume of water to prevent azide buildup in the plumbing.
   g. Avoid contact of Detection Reagents I and II with skin and mucous membranes. Wash with water if these reagents come into contact with skin. If spills of these reagents occur, dilute with water before wiping dry.

4. Storage and handling requirements:

   a. Probe Reagent Tubes must be stored in the foil pouches at 2° - 8°C. The Probe Reagent Tubes are stable in the unopened pouches until the expiration date indicated. Once opened, the pouch should be resealed and the tubes should be used within two months and prior to the expiration date.
   b. Other reagents used in the ACCUPROBE HISTOPLASMA CAPSULATUM CULTURE IDENTIFICATION TEST may be stored between 2° - 25°C and are stable until the expiration date indicated.
   c. DO NOT FREEZE THE REAGENTS.

5. Sample collection and preparation: The ACCUPROBE HISTOPLASMA CAPSULATUM CULTURE IDENTIFICATION TEST is designed to determine the identity of *H. capsulatum* isolated from culture. Colonies may be tested as soon as growth is visible. **Yeast colonies should be no more than one week old from the time growth is first observed. Filamentous colonies should be no more than one month old from the time growth is first observed. Sporulation is not necessary.**

   a. **Solid Media Method.** Growth from appropriate solid media such as Sabouraud Dextrose, Brain Heart Infusion, Mycobiotic (Mycosel), Inhibitory Mold Agar, Cottonseed Agar, Corn Meal Agar, Yeast Nitrogen Base Agar or 5% Sheep Blood Agar suggestive of *H. capsulatum* may be tested.

      (1) Growth can be removed with a 1 µL disposable plastic loop, a wire loop, a disposable plastic needle, or an applicator stick. Swabs should not be used due to the small volume of liquid in which the cells are subsequently resuspended.
      (2) Avoid taking large amounts of the solid media with the cells.
      (3) A 1-2 mm^2 size sample of yeast or filamentous growth is recommended for the test.
      (4) The operator may elect to inoculate another culture plate at this time to confirm the purity of the isolate.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

b. **Broth Culture Method.** Growth in Brain Heart Infusion broth with turbidity equivalent to or greater than a McFarland 1 Nephelometer Standard may be tested with the ACCUPROBE HISTOPLASMA CAPSULATUM CULTURE IDENTIFICATION TEST. Pipette a 100 µL sample from the well-mixed broth suspension into the Lysing Reagent Tube as described below.

6. Materials provided:

ACCUPROBE HISTOPLASMA CAPSULATUM CULTURE IDENTIFICATION TEST

Cat. No. 2910	**20 Tests**
Probe Reagent	10 x 2 tubes
Lysing Reagent	1 x 20 tubes

7. Materials required but not provided:

a. 1 µL plastic sterile inoculating loops, wire loops, plastic needles, or applicator sticks for selecting colonies
b. Control culture strains
c. Water bath or heating block (60° ± 1°C)
d. Water bath or heating block (95° ± 5°C)
e. Micropipettes (100 µL, 300 µL)
f. Re-pipettor (100 µL, 300 µL)
g. Vortex mixer
h. AVAILABLE FROM GEN-PROBE:

(1) GEN-PROBE Luminometer: LEADER or ACCULDR (formerly PAL)
(2) GEN-PROBE Sonicator or equivalent
(3) ACCUPROBE CULTURE IDENTIFICATION REAGENT KIT (Cat.No.2800)
(4) GEN-PROBE DETECTION REAGENT KIT (Cat.No.1791)
(5) GEN-PROBE Heating Block (Cat.No.2775)
(6) GEN-PROBE Sonicator Rack (Cat.No.1357)

8. Test procedure:

a. Equipment Preparation

(1) For optimal transfer of sonic energy, water must be thoroughly degassed according to the following procedure:

(a) Add enough water to fill the sonicator bath to within 1/2 inch of the top of the tank.
(b) Run the sonicator for 15 minutes to thoroughly degas the water. Sonicator should be used within a Biological Safety Cabinet.
(c) Adjust one heating block or water bath to 60° ± 1°C and another heating block or water bath to 95° ± 5°C.

(2) Prepare the Gen-Probe luminometer for operation. Make sure there is sufficient volume of Detection Reagents I and II to complete the tests.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

b. Controls: Positive and negative control strains should be tested routinely with each batch of test run. A culture of *H. capsulatum* (ATCC #38904, UTMB 3537) may be used as the positive control, while a culture of *Blastomyces dermatitidis* (ATCC #60916, UTMB 3538) may be used as the negative control.

c. Sample Preparation

(1) Label a sufficient number of Lysing Reagent Tubes to test the culture isolates and/or controls. Remove and retain the caps.
(2) Pipette 100 µL of Reagent 1 (Lysis Reagent) and 100 µL of Reagent 2 (Hybridization Buffer) into all Lysing Reagent Tubes. **If broth cultures are to be tested, do not add Reagent 1 to the Lysing Reagent Tubes.**
(3) Transfer the sample from the solid media or 100 µL of a well mixed broth culture into the labeled Lysing Reagent Tubes as described in the SAMPLE COLLECTION AND PREPARATION Section. Twirl the loop, needle or stick in the Reagent 1 and Reagent 2 diluent mixture to remove the cells if testing growth from solid media.
(4) Recap the Lysing Reagent Tubes and briefly VORTEX .

d. Sample Lysis

(1) Push the Lysing Reagent Tubes through the Sonicator Rack so that the reaction mixture in the bottom of the tube is submerged but the caps are above the water. Place Sonicator Rack on water bath sonicator. DO NOT ALLOW THE TUBES TO TOUCH THE BOTTOM OR SIDES OF THE SONICATOR.
(2) Sonicate for 15 minutes.
(3) Place the Lysing Reagent Tubes, containing the sonicated organisms in a heating block or water bath for 10 minutes at 95° ± 5°C.
(4) Carefully remove the Lysing Reagent Tubes from the heating block or water bath.

e. Hybridization

(1) Open the foil pouch by cutting evenly across the top of the pouch. Remove enough Probe Reagent Tubes to test the culture isolates and/or controls. Reseal the pouch by folding the opened edge over several times and securing with adhesive tape or a clip. **Leave the desiccant pillow in the pouch.**
(2) Label a sufficient number of Probe Reagent Tubes to test the culture isolates and/or controls. Remove and retain caps.
(3) Pipette 100 µL of the lysed specimens from the Lysing Reagent Tubes into the corresponding Probe Reagent Tubes.
(4) Recap the Probe Reagent Tubes and incubate for 15 minutes at 60° ± 1°C in a water bath or heating block.

f. Selection

(1) Remove the Probe Reagent Tubes from the water bath. Remove and retain the caps. Pipette 300 µL of Reagent 3 (Selection Reagent) into each tube. If a heating block is used, Reagent 3 may be pipetted directly into the tubes. Recap the tubes and VORTEX them to mix completely.
(2) Incubate the Probe Reagent Tubes for 5 minutes at 60° ± 1°C in a water bath or heating block.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

(3) Remove the Probe Reagent Tubes from the water bath or heating block and leave them at room temperature for at least 5 minutes. Remove and discard the caps. **Read the results in the luminometer within 1 hour after removing from water bath or heating block.**

g. Detection

(1) Select the appropriate protocol from the menu of the luminometer software.
(2) Using a damp tissue or paper towel, wipe each tube to ensure that no residue is present on the outside of the tube and insert the tubes into the luminometer according to the instrument directions.
(3) When the analysis is complete, remove the tube(s) from the luminometer.

9. Procedural notes:

a. REAGENTS: Reagent 2 (Hybridization Buffer) may precipitate. Warming and mixing the solution at 35° - 60° will dissolve the precipitate.
b. TEMPERATURE: The Hybridization and Selection reactions are temperature dependent. Therefore, it is imperative that the water bath or heating block is maintained within the specified temperature range.
c. TIME: The Hybridization and Selection reactions are time dependent. Hybridize at least 15 minutes but no more than 20 minutes. Incubate the Probe Reagent Tubes during the SELECTION Step for at least 5 minutes but no more than 6 minutes.
d. WATER BATH: The level of water in the water bath should be maintained to ensure that the Lysing Reagent Tubes are submerged up to, but not above, the level of the sealing ring. It should also be ensured that the entire liquid reaction volume in the Probe Reagent Tubes is submerged.
e. VORTEXING: It is critical to have a homogeneous mixture during the SAMPLE PREPARATION and SELECTION Steps, specifically after the addition of cells to Reagents 1 and 2 after the addition of Reagent 3.
f. TROUBLE-SHOOTING:

(1) Elevated negative control values (*B. dermatitidis* ATCC #60916) greater than 20,000 RLU (Relative Light Units) in the LEADER or 600 PLU (Photometric Light Units) in the ACCULDR (formerly PAL) can be caused by insufficient mixing after adding Reagent 3 (Selection Reagent) or by testing mixed cultures. Because mixed cultures can occur, a portion of the growth may be streaked onto the appropriate agar medium and incubated to check for multiple colony types.
(2) Low positive control valves (*H. capsulatum* ATCC #38904) less than 50,000 RLU in the LEADER or 1,500 PLU in the ACCULDR (formerly PAL) can be caused by insufficient cell numbers, improper sonication or by testing mixed or aged cultures. Because mixed cultures can occur, a portion of the growth may be streaked onto the appropriate agar medium and incubated to check for multiple colony types.

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

10. Results:

a. Interpretation of Results: The results of the ACCUPROBE HISTOPLASMA CAPSULATUM CULTURE IDENTIFICATION TEST are based on the following cut-off values. Samples producing signals greater than or equal to these cut-off values are considered positive. Signals less than these cut-off values are considered negative. Results in repeat ranges should be repeated.

	ACCULDR™ (formerly PAL)	**LEADER™**
Cut-off value	1,500 PLU	50,000 RLU
Repeat range	1,200-1,499 PLU	40,000-49,999 RLU

b. Quality Control and Acceptability of Results: Negative control (e.g., *B. dermatitidis*, ATCC #60916) and positive control (e.g., *H. capsulatum*, ATCC #38904) should satisfy the following values:

	ACCULDR™ (formerly PAL)	**LEADER™**
Negative control	<600 PLU	<20,000 RLU
Positive control	>1,500 PLU	>50,000 RLU

11. Limitations: This method has been tested using fresh growth from solid media and from broth cultures listed in the SAMPLE COLLECTION AND PREPARATION Section. The efficacy of this test has not been demonstrated on direct clinical specimens (e.g., respiratory specimens or CSF). Results from the ACCUPROBE HISTOPLASMA CAPSULATUM CULTURE IDENTIFICATION TEST should be interpreted in conjunction with other laboratory and clinical data available to the clinician.

12. Expected values: The ACCUPROBE HISTOPLASMA CAPSULATUM CULTURE IDENTIFICATION TEST was compared to standard culture identification methods at two sites using a total of 281 isolates: 114 isolates of *H. capsulatum* (12 isolates were tested in both the yeast and the filamentous phase), 26 isolates of 3 other genera of dimorphic fungi (18 *Blastomyces dermatitidis*, 6 *Coccidioides immitis*, 2 *Sporothrix schenckii)* and 141 other isolates representing 73 genera. Standard culture identification is dependent on microscopic and macroscopic characteristics of the colony, sporulation characteristic and exoantigen test. The isolates were categorized as either positive (>50,000 RLU) or negative (<50,000 RLU). The range of observations for negative cultures was 703 to 23,846 RLU and 180,071 to 720,550 RLU for positive cultures. A comparison of these results to standard culture identifications methods is shown below.

ACCUPROBE/CULTURE IDENTIFICATION

ACCUPROBE Culture	**Pos Pos**	**Pos Neg**	**Neg Pos**	**Neg Neg**	**Sensitivity/ Specificity**	**Percent Agreement**
Site 1	60	0	0	40	100%/100%	100%
Site 2	54	0	0	127	100%/100%	100%
Total	**114**	**0**	**0**	**167**	**100%/100%**	**100%**

II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

13. Performance characteristics:

a. Within-Run Precision: The within-run precision of the ACCUPROBE HISTOPLASMA CAPSULATUM CULTURE IDENTIFICATION TEST was calculated by assaying two concentrations of ribosomal RNA isolated from *H. capsulatum* using 10 replicates in a single assay.

Sample	**A**	**B**
Number of Replicates	10	10
Mean Response	48,941	103,783
Standard Deviation	1,684	2,589
Coefficient of Variation	3.4%	2.5%

b. Between-Run Precision: The between-run precision was calculated by assaying the same two concentrations of *H. capsulatum* ribosomal RNA using single determinations in 15 consecutive runs.

Sample	**A**	**B**
Number of Replicates	15	15
Mean Response	54,965	100,201
Standard Deviation	6,373	5,548
Coefficient of Variation	11.6%	5.5%

c. Specificity: A total of 96 ATCC culture isolates were evaluated using the ACCUPROBE HISTOPLASMA CAPSULATUM CULTURE IDENTIFICATION TEST. These isolates represented a total of 68 species from 41 genera. Six isolates of *Histoplasma capsulatum* (filamentous and yeast phase), 6 isolates of 3 other dimorphic fungi (*Blastomyces dermatitidis, Coccidioides immitis, Sporothrix schenckii*) and 84 isolates of 37 other genera representing a phylogenetic cross-section of organisms were evaluated using the ACCUPROBE HISTOPLASMA CAPSULATUM CULTURE IDENTIFICATION TEST. All *Histoplasma capsulatum* isolates tested produced a positive result using the ACCUPROBE HISTOPLASMA CULTURE IDENTIFICATION TEST. The representative phylogenetic cross-section isolates did not react in this test.

d. Recovery: *Histoplasma capsulatum* ribosomal RNA at concentrations ranging from 0.02 µg to 0.06 µg per test was assayed in the presence of ribosomal RNA of either *Blastomyces dermatitidis* or *Candida albicans* ranging from 0.0045 µg (equivalent to 10^5 cells) to 0.45 µg (equivalent to 10^7 cells). There was no interference of *Histoplasma capsulatum* signal observed and the other organisms present did not react using the ACCUPROBE HISTOPLASMA CAPSULATUM CULTURE IDENTIFICATION TEST.

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II. **TECHNIQUES USED IN MOULD IDENTIFICATION**: (cont'd)

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III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**:

A. Media Used for Sporulation and/or Subculture of Moulds

1. Brain Heart Infusion Agar

a. Formulation:

Bacto Brain Heart Infusion Agar (Difco)	52 g
Distilled water	1000 ml

b. Preparation:

(1) Add agar to water. Mix.
(2) Heat to boiling to dissolve medium completely.
(3) Dispense 20 ml into clean 25 x 150 mm screw cap tubes.
(4) Sterilize by autoclaving for 15 minutes at 15 psi.
(5) Slant tubes on cooling racks until agar hardens.
(6) Tighten caps and label tubes.

c. Storage conditions: 4°C refrigerator
d. Shelf life: 3 months
e. Quality control:

(1) Sterility
(2) Performance: Good growth of *Cryptococcus neoformans*.
(3) This medium is also available commercially which, in this case, is exempt of QC by wuser (NCCLS).

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

2. Histoplasma Yeast Phase Medium

a. Formulation:

Bacto Brain Heart Infusion Agar (Difco)	5.2 g
Glutamine (0.2 M) stock, frozen	1.0 ml
Sheep blood	5.0 ml
Distilled water	0.0 ml

b. Preparation:

(1) Agar medium

(a) Mix BHI agar and water.
(b) Sterilize by autoclaving for 15 minutes at 15 psi.
(c) When the medium cools, add 1.0 ml of sterile stock glutamine and 5.0 ml of sterile sheep blood. Mix.
(d) Dispense 7 ml aliquots into sterile 16 x 125 mm screw cap tubes.
(e) Slant tubes on cooling racks until agar hardens.
(f) Tighten caps and label tubes.

(2) Glutamine stock 0.2 M

(a) Add 0.292 g of glutamine to 10 ml of distilled water.
(b) Filter sterilize.
(c) Dispense 1.0 ml aliquots into tubes suitable for freezer storage.
(d) Tighten caps, label tubes with preparation date.
(e) Store at -70ºC.

c. Storage conditions: 4°C refrigerator
d. Shelf life: 3 months
e. Quality control:

(1) Sterility
(2) Performance: Conversion of *Histoplasma* mould phase to yeast phase.

3. Kelley's Agar

a. Formulation:

Glucose	10 g
Bacto peptone (Difco)	10 g
Sodium Chloride	5 g
Beef extract	3 g
Defribrinated sheep blood	5 g
Agar	15 g
Distilled water	995 ml

b. Preparation:

(1) Mix glucose, peptone, sodium chloride, beef extract, sheep blood and agar in 995 ml of distilled water.
(2) Boil to dissolve.
(3) Dispense 10 ml aliquots in 16 x 125 mm screw cap tubes.
(4) Sterilize by autoclaving 10 minutes at 15 psi.
(5) Slant tubes on rack until agar hardens.
(6) Tighten caps and label tubes.

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

c. Storage conditions: 4ºC refrigerator
d. Shelf life: 3 months
e. Quality control:

(1) Sterility
(2) Performance: Conversion of *Blastomyces* mould phase to yeast phase.

4. Malt Extract Agar

a. Formulation:

Malt extract	20 g
Peptone	1 g
Glucose	20 g
Agar	20 g
Distilled water	1000 ml

b. Preparation:

(1) Add all ingredients to distilled water.
(2) Boil to dissolve.
(3) Sterilize by autoclaving for 15 minutes at 15 psi.
(4) Dispense 15 ml aliquots into sterile 15 x 100 Petri plates.
(5) Label plates and bag to reduce contamination and dehydration.

c. Storage conditions: 4°C refrigerator
d. Shelf life: 6 weeks
e. Quality control:

(1) Sterility
(2) Performance: Good growth of *Candida albicans*

5. Mycosel

a. Formulation:

Mycosel agar (BBL)	36.6 g
Distilled water	1000 ml

b. Preparation:

(1) Add agar to water.
(2) Boil to dissolve medium completely.
(3) Adjust the pH to 6.5 + 0.2.
(4) Dispense 20 ml aliquots into clean 25 x 150 mm screw cap tubes.
(5) Sterilize by autoclaving for 15 minutes at 15 psi.
(6) Slant tubes on cooling racks until agar hardens.
(7) Tighten caps and label tubes.

c. Storage conditions: 4°C refrigerator
d. Shelf life: 3 months

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

e. Quality control:

(1) Sterility
(2) Performance:

(a) Poor or no growth of *Staphylococcus aureus*.
(b) Poor or no growth of *Aspergillus flavus*.
(c) Good growth of *Trichophyton mentagrophytes*.
(d) When the medium is obtained commercially prepared, no QC is to be performed by the user (NCCLS).

6. Oatmeal Agar Medium - Weitzman and Silva-Hutner: For perfect state of dermatophytes and other pathogenic fungi. Sabourodia 5:335-340.

a. Formulation:

$MgSO_4$ $7H_2O$	1 g
KH_2PO_4	1.5 g
$NaNO_3$	1 g
Oatmeal	10 g
Agar	18 g
Distilled water	1000 ml

b. Preparation:

(1) Combine ingredients.
(2) Adjust pH with NaOH to 5.6.
(3) Autoclave at 121ºC for 20 minutes.

c. Storage conditions: 4°C refrigerator
d. Shelf life: 3 months
e. Quality control:

(1) Sterility
(2) Performance: Ascospores produced by crossing dermatophyte tester strains.

7. Potato Dextrose Agar

a. Formulation:

Potatoes	100 g
Glucose	5 g
Agar	7.5 g
Distilled water	500 ml

b. Preparation:

(1) Peel and dice 100 g of potatoes and add to 500 ml of distilled water.
(2) Autoclave 10 minutes at 15 psi to make the potato infusion.
(3) Filter infusion through gauze.
(4) Add 7.5 g of agar.
(5) Boil to dissolve the agar.
(6) Add 5 g of glucose.
(7) Bring the total volume to 500 ml with distilled water if necessary.
(8) For tubes: Dispense 5 ml into 16 x 125 mm screw cap tubes. Sterilize by autoclaving for 15 minutes at 15 psi. Slant tubes until agar hardens. Label tubes and tighten caps.

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

(9) For plates: Sterilize 15 minutes at 15 psi. Dispense 18 ml into 15 x 100 mm sterile Petri plates. Label plates. Bag to reduce contamination and dehydration.

c. Storage conditions: 4°C refrigerator
d. Shelf life:

(1) Plates: 6 weeks in bags.
(2) Tubes: 3 months

e. Quality control:

(1) Sterility
(2) Performance: Good growth of *Candida albicans*.

8. Potato Sucrose Agar

a. Formulation:

Potatoes	100 g
Sucrose	10 g
Agar	10 g
Distilled water	500 ml

b. Preparation:

(1) Peel and dice 100 g of potatoes. Add to 500 ml of distilled water.
(2) Autoclave 10 minutes at 15 psi to make the potato infusion.
(3) Filter infusion through gauze.
(4) Bring volume to 500 ml with distilled water if necessary.
(5) Add agar. Boil to dissolve.
(6) Add sucrose.
(7) For tubes: Dispense 5 ml aliquots into 16 x 125 screw cap tubes. Sterilize by autoclaving for 15 minutes at 15 psi. Slant tubes until agar hardens. Label tubes and tighten caps.
(8) For plates: Sterilize by autoclaving for 15 minutes at 15 psi. Dispense 18 ml into sterile 15 x 100 mm Petri plates. Label plates for bag for storage.

c. Storage conditions: 4°C
d. Shelf life:

(1) Plates: 6 weeks in bags
(2) Tubes: 3 months

e. Quality control:

(1) Sterility
(2) Performance: Good growth of *Candida albicans*

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

9. Rice Grains

   a. Formulation:

Unfortified white rice grains	8 g
Distilled water	25 ml

   b. Preparation:

      (1) Place 8 g of rice grains and 25 ml of distilled water into a 125 ml screw cap flask.
      (2) Sterilize by autoclaving for 15 minutes at 15 psi.

   c. Storage conditions: 4°C refrigerator
   d. Shelf life: 1 week
   e. Quality control:

      (1) Sterility: Not necessary prior to use.
      (2) Performance:

         (a) Good growth of *Microsporum canis*.
         (b) No growth of Microsporum audouinii.

10. Sabouraud Dextrose Agar (Emmons' modification)

   a. Formulation:

Sabouraud dextrose agar	20 g
Distilled water	1000 ml

   b. Preparation:

      (1) Add agar to water.
      (2) Boil to dissolve agar.
      (3) Adjust pH to 6.5.
      (4) Tubes and plates:

         (a) For tubes: Dispense 15 ml aliquots into clean 25 x 150 screw cap tubes. Sterilize by autoclaving for 15 minutes at 15 psi. Slant tubes on cooling racks until agar hardens. Tighten caps and label tubes.
         (b) For plates: Sterilize by autoclaving for 15 minutes at 15 psi. Dispense 20 ml into sterile 15 x 100 Petri plates. Label plates, and bag to reduce contamination and dehydration.

   c. Storage conditions: 4°C
   d. Shelf life: 3 months
   e. Quality control:

      (1) Sterility
      (2) Performance: Good growth of *Candida albicans*.
      (3) When the medium is obtained prepared from a commerical source, no QC is to be performed by user (NCCLS).

11. Sabouraud's Agar Plus Chloramphenicol

   a. Formulation:

Sabouraud dextrose agar	50 g
Chloramphenicol (assay powder)	0.05 g
Distilled water	1000 ml

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

b. Preparation:

(1) Dissolve 0.05 g of chloramphenicol in 10 ml of 95% ethanol.
(2) Add agar to water.
(3) Boil to dissolve agar.
(4) Add chloramphenicol.
(5) Sterilize by autoclaving for 10 minutes at 15 psi.
(6) Dispense 20 ml aliquots into sterile 15 x 100 mm Petri plates.

c. Storage conditions: 4°C refrigerator
d. Shelf life: 3 months
e. Quality control:

(1) Sterility
(2) Performance:

(a) Poor or no growth of *Staphylococcus aureus*.
(b) Good growth of *Candida albicans*.

12. Sabouraud Dextrose Broth

a. Formulation:

Neopeptone	10 g
Glucose	20 g
Distilled water	1000 ml

b. Preparation:

(1) Add neopeptone and glucose to water. Mix.
(2) Dispense 7.0 ml aliquots into 16 x 125 mm screw cap tubes.
(3) Sterilize by autoclaving for 10 minutes at 15 psi.

c. Storage conditions: 4°C
d. Shelf life: 3 months
e. Quality control:

(1) Sterility
(2) Performance: Good growth of *Cryptococcus neoformans*.

13. V-8 Juice Agar - to promote sporulation, indicate colored fungi, yeast, and some ascomycetes

a. Formulation:

V-8 juice	100 ml
$CaCO_3$	2.5 g
Agar	18 g
Distilled water	880 ml

b. Preparation:

(1) Add agar to 800 ml of water in one flask.
(2) Boil to dissolve agar completely.
(3) Add V-8 juice, $CaCO_3$ and 80 ml of water in another flask.
(4) Adjust the juice-yeast solution to pH 6.8.
(5) Heat the juice-yeast solution for 10 minutes in flowing steam.
(6) Readjust pH to 6.8 if necessary.

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

(7) Mix the agar solution with the juice solution.
(8) Dispense 7 ml aliquots into 16 x 125 mm screw cap tubes.
(9) Sterilize by autoclaving for 15 minutes at 15 psi.
(10) Slant tubes on cooling racks until the agar hardens.
(11) Tighten caps and label tubes.

c. Storage conditions: 4°C refrigerator
d. Shelf life: 3 months
e. Quality control:

(1) Sterility
(2) Performance: Abundant production of ascospores by *Saccharomyces cerevisiae*

14. Yeast Extract-Phosphate Agar

a. Formulation:

(1)	Solution A:	Yeast extract	1.0 gm
		Agar	15.0 gm
		Distilled water	1 liter
(2)	Solution B:	Disodium phosphate, anhydrous	4.0 gm
		Monopotassium phosphate	6.0 gm
		Distilled water	10.0 ml
(3)	Solution C:	Ammonium hydroxide, concentrated	1.0 ml

b. Preparation:

(1) Solution A

(a) Mix reagents.
(b) Bring solution to a boil.

(2) Solution B

(a) Mix reagents.
(b) Adjust pH to 6.0 with either 1 *N* Hcl or 1 *N* NaOH.

(3) Add 2.0 ml of soluion B to solution A.
(4) Autoclave for 15 minutes at 15 psi.
(5) Dispense into sterile Petri dishes (35 ml per dish).

c. Storage conditions: 4°C
d. Shelf life: 14 days
e. Quality control:

(1) Sterility
(2) pH reaction of 6.0

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

(3) Performance

Fungus	Incubation temperature	Result
Blastomyces dermatitidis	25°C	Good growth

f. Comments: Approximately 0.1-0.5 ml of the contaminated specimen that has been concentrated is placed onto the medium. One drop (0.05 ml) of solution C is placed onto the medium surface away from the sediment. The ammonium hydroxide will diffuse throughout the medium. Cycloheximide cannot be incorporated into the medium because the ammonium hydroxide will inactivate it. Antibacterial agents such as chloramphenicol can be added to the medium.

15. Yeast Extract-Malt Extract (YM) Agar

a. Formulation:

Yeast extract	3.0 gm
Malt extract	3.0 gm
Peptone	5.0 gm
Glucose	10.0 gm
Agar	15.0 gm
Distilled water	1 liter

b. Preparation:

(1) Mix reagents.
(2) Bring to a boil.
(3) Dispense 7.0-ml aliquots into 16 x 125 mm test tubes.
(4) Autoclave for 15 minutes at 15 psi.
(5) Slant the test tubes.

c. Storage conditions: 4°C
d. Shelf life:

(1) 30 days in test tubes
(2) 14 days in Petri dishes

e. Quality control:

(1) Sterility
(2) Performance

Fungus	Incubation temperature	Result
Saccharomyces cerevisiae	25°C	Good growth

f. Comments: The pH can be adjusted to 3.7 with dilute hydrochloric acid. Acidified YM agar or broth can be used to purify yeast cultures that have a bacterial contaminant.

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

16. Water Agar, 2%

a. Formulation:

Agar	20.0 gm
Distilled water	1 liter

b. Preparation:

(1) Mix reagents.
(2) Bring to a boil.
(3) Dispense 15.0-ml aliquots into 25 x 125 mm test tubes.
(4) Autoclave for 15 minutes at 15 psi.

c. Storage conditions: 4°C
d. Shelf life:

(1) 30 days in test tubes
(2) 14 days in Petri dishes

e. Quality control:

(1) Sterility
(2) Performance; not necessary prior to use

f. Comments: The water agar is melted and poured into sterile Petri dishes prior to use.

17. Oatmeal Agar for the Gymnoascaceae

a. Formulation:

Tomato paste (Hunt's)	10.0 gm
Oatmeal (Beech-Nut for babies)	10.0 gm
Magnesium sulfate	1.0 gm
Potassium phospate	1.0 gm
Sodium nitrate	1.0
Agar	15.0 gm
Distilled water	1 liter

b. Preparation:

(1) Mix reagents.
(2) Bring to a boil.
(3) Cool, adjust pH to 5.6 with sodium hydroxide.
(4) Autoclave for 20 minutes at 15 psi.
(5) Dispense 20.0-ml aliquots into sterile Petri dishes.
(6) Allow to harden.

c. Storage conditions: 4°C
d. Shelf life: 14 days
e. Quality control:

(1) Sterility
(2) pH reaction approximately 5.6
(3) Performance; not necessary prior to use

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

f. Comments: The medium is excellent for stimulating the formation of gymnothecia by members of the Gymnoascaceae. The medium is commonly used for the genera *Arthroderma* and *Nannizzia*.

18. Malt Extract Agar for *Aspergillus*

a. Formulation:

Malt extract	20.0 gm
Peptone (Difco)	1.0 gm
Glucose	20.0 gm
Agar	20.0 gm
Distilled water	1 liter

b. Preparation:

(1) Mix reagents.
(2) Bring to a boil.
(3) Dispense 7.0-ml aliquots into 16 x 125 mm test tubes.
(4) Autoclave for 15 minutes at 15 psi.
(5) Slant the test tubes.
(6) For plates, autoclave medium in a flask.

(a) Aseptically dispense 25.0-ml aliquots into sterile Petri dishes.
(b) Allow medium to harden.

c. Storage conditions: 4°C
d. Shelf life:

(1) 30 days in test tubes
(2) 14 days in Petri dishes

e. Quality control:

(1) Sterility
(2) Performance

Fungus	Incubation temperature	Result
Aspergillus flavus	25°C	Rapid growth, yellowish-green colonies

19. Malt Extract (ME) Agar

a. Formulation:

Malt extract	20.0 gm
Agar	12.0 gm
Distilled water	400.0 ml

b. Preparation:

(1) Mix agar and distilled water.
(2) Bring to a boil.
(3) Cool, ad malt extract and mix.
(4) Dispense 7.5-ml aliquots into 16 x 125 mm test tubes.
(5) Autoclave for 15 minutes at 15 psi.
(6) Slant the test tubes.

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

c. Storage conditions: 4°C
d. Shelf life:

(1) 30 days in test tubes
(2) 14 days in Petri dishes

e. Quality control:

(1) Sterility
(2) Performance

Fungus	Incubation temperature	Result
Saccharomyces cerevisiae	25°C	Good growth

f. Comments: The medium can be acidified with hydrochloric acid to a pH reaction of 3.7 if it is to be used for purifying bacterially contaminated yeast cultures.

20. Hay Infusion Agar

a. Formulation:

Decomposing hay	50.0 gm
Dipotassium phosphate	2.0 gm
Agar	15.0 gm
Tap wate r	1 liter

b. Preparation:

(1) Mix hay and tap water.
(2) Autoclave for 30 minutes at 15 psi.
(3) Filter through cheesecloth.
(4) Add reagents to the hay infusion.
(5) Cool and adjust pH to approximately 6.2.
(6) Bring to a boil.
(7) Dispense 7.0-ml aliquots into 25 x 125 mm test tubes.
(8) Autoclave for 15 minutes at 15 psi.
(9) Slat the test tubes.

c. Storage conditions: 4°C
d. Shelf life:

(1) 30 days in test tubes
(2) 14 days in Petri dishes

e. Quality control:

(1) Sterility
(2) pH reaction approximately 6.2.
(3) Performance; not necessary prior to use

f. Comments: The medium is often used to enhance the development of conidia or spores.

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

21. Czapek-Dox-Solution Agar for *Aspergillus*

a. Formulation:

Sodium nitrate	3.0 gm
Dipotassium phosphate	1.0 gm
Magnesium sulfate	0.5 gm
Potassium chloride	0.5 gm
Ferrous sulfate	0.01 gm
Glucose	30.0 gm
Agar	15.0 gm
Distilled water	1 liter

b. Preparation:

(1) Mix the reagents.
(2) Bring to a boil.
(3) Dispense 7.0-ml aliquots into 16 x 125 mm test tubes.
(4) Autoclave for 15 minutes at 15 psi.
(5) Slant the test tubes.
(6) For plates, autoclave medium in a flask.

(a) Aseptically dispense 25.0-ml aliquots into sterile Petri dishes.
(b) Allow medium to harden.

c. Storage conditions: 4°C
d. Shelf life:

(1) 30 days in test tubes
(2) 14 days in Petri dishes

e. Quality control:

(1) Sterility
(2) Performance

Fungus	Incubation temperature	Result
Aspergillus flavus	25°C	Good growth, colonies thin, at first yellow, becoming green

22. Czapek-Dox-Solution Agar

a. Formulation:

Magnesium sulfate	0.5 gm
Potassium chloride	0.5 gm
Dipotassium phosphate	1.0 gm
Ferrous sulfate	0.01 gm
Sodium nitrate	3.0 gm
Sucrose	30.0 gm
Agar	15.0 gm
Distilled water	1 liter

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

b. Preparation:

(1) Mix reagents.
(2) Bring to a boil.
(3) Dispense 7.0-ml aliquots into 16 x 125 mm test tubes.
(4) Autoclave for 15 minutes at 15 psi.
(5) Slant the test tubes.

c. Storage conditions: 4°C
d. Shelf life:

(1) 30 days in test tubes
(2) 14 days in Petri dishes

e. Quality control:

(1) Sterility
(2) Performance

Fungus	Incubation temperature	Result
Aspergillus flavus	25°C	Good growth, yellow to green colony

23. Cereal Agar for Ascocarp Production in the Gymnoascaceae

a. Formulation:

Precooked mixed cereal	10.0 gm
Dipotassium phosphate	1.5 gm
Magnesium sulfate	1.0 gm
Sodium nitrate	1.0
Agar	18.0 gm
Distilled water	1 liter

b. Preparation:

(1) Mix reagents.
(2) Bring to a boil.
(3) Cool; adjust pH to 5.6.
(4) Autoclave for 15 minutes at 15 psi.
(5) Dispense 15.0-ml aliquots into sterile Petri dishes.
(6) Let the medium harden.

c. Storage conditions: 4°C
d. Shelf life: 14 days
e. Quality control:

(1) Sterility
(2) pH reaction approximately 5.6
(3) Performance; not necessary prior to use

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

24. Cereal Agar for Hyphomycetes

a. Formulation:

Precooked mixed cereal	100.0 gm
Agar	15.0 gm
Distilled water	1 liter

b. Preparation:

(1) Mix reagents
(2) Bring to a boil.
(3) Dispense 7.0-ml aliquots into 16 x 125 mm test tubes.
(4) Autoclave for 10 minutes at 10 psi.
(5) Slant the test tubes.

c. Storage conditions: 4°C
d. Shelf life: 30 days
e. Quality control:

(1) Sterility
(2) Performance

Fungus	Incubation temperature	Result
Trichophyton mentagrophytes	25°C	Good production of conidia

f. Comments: Cereal agar can be supplemented with 50.0 mg of chloramphenicol per liter if bacterial contamination may be a problem.

B. Reagents Used for Mould Identification:

1. Hexachlorocyclohexane (Mite Control)

a. Formulation:

hexachlorocyclohexane	0.01 gm
any medium	1.0 liter

b. Preparation: Add 0.01 gm of hexachlorocyclohexane to 1.0 liter of any mycological media prior to autoclaving. Prepare as normal.

2. Lactophenol and Lactophenol Cotton Blue Mounting Media

a. Formulation:

(1)	Lactophenol	phenol (concentrated)	20.0 ml
		lactic acid	20.0 ml
		glycerol	40.0 ml
		distilled water	20.0 ml
(2)	Lacto-phenol cotton blue	phenol (concentrated)	20.0 ml
		lactic acid	20.0 ml
		glycerol	40.0 ml
		cotton blue	0.05 gm
		distilled water	20.0 ml

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

b. Preparation:

(1) Mix phenol, lactic acid, glycerol and water in a 250 ml screw cap bottle.
(2) For lacto-phenol cotton blue, dissolve 0.05 gm of cotton blue in 20 ml of water. Then add phenol, lactic acid and glycerol. Store in 250 ml screw cap bottle.

3. Mounting Media

a. Wetting Agent for use with Lacto-fuchsin or PVA

95% Ethanol	50 ml
Acetone	25 ml
85% lactic acid	25 ml

(1) Let a drop of wetting agent fall on the material to be mounted while it is still on the transfer needle. Drain by touching on a clean filter paper.
(2) Mount in glycerine jelly or lacto-fuchsin.
(3) Use: Fungal hyphae are hydrophobic and shed water. The wetting agent allows the stain to penetrate the hyphae and eliminates air bubbles.

b. Glycerine Jelly

Glycerine	7 g
Gelatin	1 g
Water	6 ml
Phenol	1.4 g

(1) Mix gelatin in distilled water and dissolve 2 hr or more, heating gently on low. Add glycerine and phenol crystals. Warm 15 min. until phenol dissolves, then filter through gauze. Transfer to wide mouth plastic jar and store in refrigerator.
(2) Transfer small amount for daily use to borosilicate glass balsam bottle. Heat bottle on hot plate set at low until melted. Use glass rod to dispense drop of mounting medium.
(3) This product solidifies on cooling and deteriorates on repeated melting.
(4) Quality Assurance: Discard discolored medium, or medium failing to solidify upon cooling.
(5) Use: A permanent mounting medium. Fungal hyphae are not readily distorted. (Ref.: Ainsworth, G: Ainsworth & Bisby's Dictionary of the Fungi, 5th Edition, Kew, Surrey, U.K., Commonwealth Mycological Institute, p. 243, 1961.)

c. Lacto-fuchsin

Acid fuchsin	0.1 g
85% Lactic acid	100 ml

III. **MEDIA AND REAGENTS USED IN MOULD IDENTIFICATION**: (cont'd)

Use: Mounts made with lacto-fuchsin are similar to those made with lactophenol cotton blue but are superior in that cell walls stand out more clearly since the refractive index is further from that of the cell walls of hyaline fungi, and staining is more rapid. (Ref.: Carmichael, JW: Lacto-fuchsin: a new medium for mounting fungi. Mycologia 4:611, 1955)

d. Polyvinyl alcohol (PVA)

Polyvinyl alcohol (Sigma Chemicals)	1.66 g
Lactic acid	10.0 ml
Glycerine	1.0 ml
Water	10.0 ml

(1) Add PVA crystals to the water. When dissolved, add lactic acid, stirring vigorously. Add glycerine. Filter if necessary. Allow to sit for 24 hr to mature.
(2) Quality assurance: Discard if medium fails to harden.
(3) Use: As a colorless permanent mounting medium that does not discolor when exposed to sunlight. It requires hardening for 10 min at 40°C before examination under oil and 3-5 days at room temperature or 36 hr at 40°C to completely harden. (Ref.: Salmon, J: A new polyvinyl alcohol mounting medium. The Microscope 10:66-68, 1954)

e. Wetting Agent for use with Glycerine Jelly

95% Ethanol	50 ml
Acetone	25 ml
Glycerol	25 ml

Ref: L. Sigler/A. Flis UAMH July 26, 1990

IV. **AEROBIC ACTINOMYCETES IDENTIFICATION**:

Separation of genera and species of aerobic actinomycetes has been tradiitonally based upon physiological tests, staining reactions (modified acid-fast stain), growth in slide culture, andlysozyme resistance.

Culture and identificaiton of anaerobic actinomycetes (e.g., Actinomyces sp.) will be conducted in the Bacteriology Laboratory anaerobe section.

A. Identification of an Aerobic Actinomycete

1. Macroscopic Morphology

a. When obvious growth of the subculture is observed (10-14 days, R°; 4-8 days, 35°C), it sould be examined for the presence of aerial mycelium which is best done under the dissecting microscope.
b. If the colony surface is covered with aerial hyphae, it can be assumed that the isolate is an actinomycete and physiologic tests for its identification may be set up.
c. If the colony surface shows no or very little aerial hyphae, slide cultures are set up according to the method in Section ?. Three media are used: BHI, potato dextrose, and Sabouraud agars.

2. Microscopic Morphology

a. Slide cultures are incubated at 30-35°C for approximately a week at which time they are examined for development of aerial hyphae. Occasionally cultures may have to be held for two to four weeksbefore deciding whether or not the organism is an actinomycete.
b. Interpretation of slide culture:

(1) Slide cultures of the filamentous aerobic organisms in the Actinomycetales order are not as easy to interpret as are those of moulds. Extension of the vegetative filaments from the substrate to the undersurface of the glass coverslip is noted regardless of whether or not *Nocardiaceae*, *Thermoactinomycetaceae*, or *Mycobacteriaceae* family.
(2) If the organism is a *Nocardia*, *Streptomyces*, or *Actinomadura* species, within one to four weeks, the growth on the glass surface will show profuse brancing with much intertwining and anastomosis of filaments resulting in the development of a beautiful, delicate, lace-like mycelium. Careful use of the fine adjustor of the microscope will also reveal delicate aerial filaments that are "waving in the breeze."
(3) Members of the *Mycobacterium* and rhodochrous groups will not develop these aerial filaments nor do they develop a well-defined mycelium.
(4) All *Micromonospora* species as well as an occasional isolate of *Actinomadura madura*, or a *Nocardia* species, do not develop aerial filaments or a good mycelium. In such instances, the medical technologist may not be able to differentiate these organisms from those of the *Mycobacterium* and rhodochrous groups. Such isolates should be sent to a reference laboratory for identification.

III. **AEROBIC ACTINOMYCETES IDENTIFICATION**:

3. Physiologic Tests

a. At least five physiologic tests are used routinely in the clinical laboratory to identify several Nocardia species and A. madurae. The following table summarizes these tests and expected results for these actinomycetes.

	Duration of Incubation	*N. aster.*	*N. bras.*	*N. otitis-caviarum*	*S. griseus*	*A. madurae*
Lysozyme	1 wk	R[1]	R	R	S	S
Casein	2 wk	0	+	0	+/-[3]	+
Xanthine	3 wk	0	0	+	+	0
Hypoxanthine	3 wk	0	V	+	+	0
Tyrosine	4 wk	0	+	0	+	+/-
Urea Broth	4 wk	V[2]	+	V	V	0

[1]R = resistant to lysozyme, i.e. grows in presence of lysozyme
[2]V = variable
[3]+/- = usually positive; rare isolate may be negative

b. With the exception of *N. brasiliensis*, there is no difference betwen reactions on xanthine and hypoxanthine by the organisms included in this table. Some laboratories use hypoxanthine because its hydrolysis is sometimes more rapid than is that of xanthine.

c. Inoculation of casein, xanthine, hypoxanthine, and tyrosine agar plates: We recommend use of 3 or 4 compartmented petri dises (15 x 100 mm). Such plates contain space for the "unknown" isolate as well as for control cultures.

(1) Inculate the surface of each meidum with enough of the culture to cover an area of approximately 5 mm. This is done by gently spreading the inoculum with either a probe or loop.
(2) Ring plates with parafilm and incubate at room temperature for aproriate time or until hydrolysis is noted (see Table 1). Clear areas will be noted around the periphery of the inoculum if the casein has been hydrolyzed or any of the other compounds have been utilized. A duplicate set of media may be inoculated and incubated at 35°C. The room temperature tests should serve as controls for detecting difficulties from incubation at a higher than recommended temperature. Occasionally, some tests will become positive more rapidly at 35°C incubation.

d. Resistance to Lysozyme

(1) Place several fragements of the culture into a tube of control broth (glycerol broth without lysozyme).
(2) Do the same with a tube of broth containing lysozyme.
(3) Inoculate a control set of broths using a known *Steptomyces* species. This control set indicates whether or not the lysozyme is active (should have growth in control tube but not in lysozyme tube). *N. asteriodes* may be used as a positive control.

III. **AEROBIC ACTINOMYCETES IDENTIFICATION**:

(4) Incubate all tubes at room temperature until control tubes show good growth, at which time the tests may be read and discarded. An actinomycete may grow in the bottom of the control broth failing to form a pellicle. Thus, look carefully for growth before agitating tubes. Most *Nocardia* isolates will form a pellicle but examination for growth in the bottom of the tubes should be made.

4. Acid Fast Stain - Use of the acid fast stain as a means of recognizing an isolate as a *Nocardia* species has always been unreliable even when done carefully. Use of the lysozyme test should replace the study of acid fast properties for recognition of a *Nocardia* isolate. The authors recognize, however, that there are still laboratories that do not use the lysozyme test. Hence, the following technique for performing the acid fast stain on a culture is presented. Although some laboratories use themodified Kinyoun's acid fast stain, there is no reliable way to accurately control the procedure. It is for this reason that the modified Hank's acid fast stain is recommended.

   a. Control Cultures - A *Nocardia* sspecies (acid fast positive) and an isolate of *A. madurae* (non-acid fast) must be stained along with the "unknown" isolate. The control organisms must be on the same medium and of the same age as the unknown isolate. (If a *Streptomyces* species is used as a negative control, it should not be heavily sporulating isolate. Conidia of *Streptomyces* may retain the acid fast stain, thus causing difficulty in interpreting the staining reaction.)
   b. Culture Medium - DO NOT attempt to do an acid fast stain on an organism growing on Sabouraud, BHI, or blood agar. All cultures should be on a medium that enhances acid fast properties such as 7H10 or 7H11 agar.
   c. Preparation of Slides - Make at least three thin smears of each of the cultures to be stained ("unknown," controls). Allow to air dry; do not heat fix.
   d. Staining Technique: Kinyoun modified acid-fast stain. See modified Kinyoun Technique as outlined in the Specimen procdure section.

5. Additional Physiologic Tests

   a. MacConkey medium without crystal violet: Make an aqueous suspension of the culture and streak the entire plate with a 3 mm loopful. Incubate plates at 35°C for 5-11 days. *M. fortuitum* grows well on this medium; rhodochrous organisms grow poorly or not at all. The aerobic actinomycetes do not grow on this medium.

B. Aerobic Actinomycete Media

1. Casein Agar

   a. Formulation:

Skim milk powder	10 g
Distilled water	90 ml
Agar	3 g
Distilled water	97 ml

   b. Preparation:

      (1) Add skim milk powder and distilled water together in a flask.
      (2) Add agar and distilled water together in another flask.
      (3) Sterilize the solutions separately by autoclaving for 10 minutes at 121°C at 15 psi.

III. **AEROBIC ACTINOMYCETES IDENTIFICATION**:

(4) Allow the solutions to cool to 50ºC and then combine them together.
(5) Aseptically dispense 25 ml aliquots into sterile 15 x 100 mm Petri plates.
(6) Label plates and bag to reduce dehydration and contamination.

c. Storage conditions: 4°C refrigerator
d. Shelf life: 3 months
e. Quality control:

(1) Sterility
(2) Performance:

(a) Hydrolysis, indicated by a clearing of the medium under and around the inoculum by *Streptomyces griseus* and *Actinomadura madurae*.
(b) No hydrolysis of the medium by *Nocardia asteroides*.

2. Basal Medium for Hypoxanthine, Tyrosine and Xanthine Agars

a. Formulation:

Beef extract	3.0 g
Peptone	5.0 g
Agar	20.0 g
Distilled water	1000.0 ml

b. Preparation:

(1) Add beef extract, peptone, agar and water together in a flask.
(2) Heat the solution until the ingredients are dissolved.
(3) Dispense 100 ml aliquots into 250 ml flasks or bottles.
(4) Sterilize by autoclaving for 10 minutes at 121°C at 15 psi.
(5) Hold the medium at 50°C in a water bath until ready to add the hypoxanthine, tyrosine and xanthine solutions.
(6) The basal medium can also be stored at 4ºC and later remelted for use.

c. Storage conditions:

(1) 50°C water bath for immediate use
(2) 4°C refrigerator for prolonged storage.

d. Shelf life: 3 months
e. Quality control: None required except for sterility since this medium is used as an addition to other media.

3. Tyrosine Agar

a. Formulation:

Basal medium, sterile melted	100 ml
Tyrosine	0.5 g
Distilled water	10 ml

b. Preparation:

(1) Prepare a sterile tyrosine solution by adding the tyrosine to the distilled water and filter sterilizing through a 0.22 u filter.
(2) Allow the sterile melted basal medium to cool almost to solidification.

III. **AEROBIC ACTINOMYCETES IDENTIFICATION**:

(3) Add 10 ml of the sterile tyrosine solution to the flask or bottle of basal medium. Mix.
(4) Aseptically dispense 25 ml aliquots into sterile 15 x 100 mm Petri plates.
(5) Label plates and bag to reduce contamination and dehydration.

c. Storage conditions: 4°C refrigerator
d. Shelf life: 2 months
e. Quality control:

(1) Sterility
(2) Performance:

(a) Hydrolysis, indicated by a clearing of the medium under and around the inoculum, by *Streptomyces griseus* and *Actinomadura madurae*.
(b) No hydrolysis of the medium by *Nocardia asteroides*.

4. Xanthine Agar

a. Formulation:

Basal medium, sterile melted	100 ml
Xanthine	0.4 g
Distilled water	10 ml

b. Preparation:

(1) Prepare a sterile xanthine solution by adding the xanthine to the distilled water and filter sterilizing through a 0.22 u filter.
(2) Allow the sterile melted basal medium to cool almost to solidification.
(3) Add 10 ml of the sterile xanthine solution to the flask or bottle of basal medium. Mix.
(4) Aseptically dispense 25 ml aliquots into sterile 15 x 100 mm Petri plates.
(5) Label plates and bag to reduce contamination and dehydration.

c. Storage conditions: 4°C refrigerator
d. Shelf life: 3 months
e. Quality control:

(1) Sterility
(2) Performance:

(a) Hydrolysis, indicated by a clearing of the medium under and around the inoculum, by *Streptomyces griseus*.
(b) No hydrolysis of the medium by *Nocardia asteriodes* or *Actinomadura madurae*

III. **AEROBIC ACTINOMYCETES IDENTIFICATION**:

5. Hypoxanthine Agar

   a. Formulation:

Basal medium, sterile melted	100 ml
Hypoxanthine	0.5 g
Distilled water	10 ml

   b. Preparation:

      (1) Prepare a sterile hypoxanthine solution by adding the hypoxanthine to the distilled water and autoclaving for 10 minutes at 121°C.
      (2) Allow the sterile melted basal medium to cool almost to solidification.
      (3) Add 10 ml of the sterile hypoxanthine solution to the flask or bottle of basal medium. Mix.
      (4) Aseptically dispense 25 ml aliquots into sterile 15 x 100 mm Petri plates.
      (5) Label plates and bag to reduce contamination and dehydration.

   c. Storage conditions: 4°C refrigerator
   d. Shelf life: 2 months
   e. Quality control:

      (1) Sterility
      (2) Performance:

         (a) Hydrolysis, indicated by a clearing of the medium under and around the inoculum, by *Streptomyces griseus*.
         (b) No hydrolysis of the medium by *Nocardia asteriodes* or *Actinomadura madurae*

6. Urea Broth

   a. Formulation:

Dehydrated urea broth, Difco	3.87 g
Distilled water	100 ml

   b. Preparation:

      (1) Dissolve dehydrated urea broth in distilled water.
      (2) Sterilize by filtration through a 0.22 µ filter.
      (3) Aseptically dispense 4 ml aliquots into sterile 16 x 125 mm screw cap tubes.
      (4) Label plates and tighten caps for storage.

   c. Storage conditions: 4°C refrigerator
   d. Shelf life: 30 days
   e. Quality control:

      (1) Sterility
      (2) Hydrolysis, indicated by a color change of the broth from yellow to red by *Nocardia asteriodes*
      (3) No hydrolysis or color change by *Actinomadura madurae*

III. **AEROBIC ACTINOMYCETES IDENTIFICATION**:

7. Lysozyme Broth

a. Formulation:

(1)	Basal glycerol broth	Beef extract	3 g
		Peptone	5 g
		Glycerol	70 ml
		Distilled water	1000 ml
(2)	Lysozyme solution	Lysozyme	100 mg
		0.01 N Hcl	100 ml

b. Preparation:

(1) Prepare the basal glycerol broth by adding the ingredients together and mixing.
(2) Dispense 500 ml of the broth of 5 ml aliquots into 16 x 125 mm screw cap tubes.
(3) Sterilize the tubes and flask of remaining broth by authoclaving for 15 minutes at 121°C and 15 psi.
(4) Prepare the lysozyme solution by adding the lysozyme and 0.01 N HCl together.
(5) Sterilize by filtration through 0.22 µ filter.
(6) Prepare the lysozyme broth by adding 5 ml of the sterile lysozyme solution to 95 ml of the sterile basal glycerol broth. Mix well.
(7) Aseptically dispense 5 ml aliquots into sterile 16 x 125 mm screw cap tubes.
(8) Save the remaining glycerol broth to prepare more lysozyme broth.

c. Storage conditions: 4°C refrigerator
d. Shelf life:

(1) Glycerol broth - 3 months
(2) Lysozyme solution or broth - 1 week

e. Quality control:

(1) Sterility
(2) Performance:

(a) Good growth of *Nocardia asteroides* in both the basal glycerol broth control tube and the lysozyme broth..
(b) Good growth of S*trptomyces griseus* and *Actinomadura madurae* in the basal glycerol broth control and no growth in the lysozyme broth.

III. **AEROBIC ACTINOMYCETES IDENTIFICATION**:

8. McClug's Carbon-Free Broth - for the isolation of *Nocardia* from contaminated sites

   a. Formulation:

$NaNO_3$	2.0 g
K_2HPO_4	0.8 g
$MgSO_4\ 7H_2O$	0.5 g
$FeCl_3$	0.10 g
$MnCl_2\ 4H_2O$	0.008 g
$ZnSO_4$	0.002 g
Distilled H_2O	to bring volume to 1 liter

   b. Preparation:

      (1) Mix ingredients; heat solution at low temperature until ingredients dissolve (15-30 minutes).
      (2) A heavy precipitate may appear as solution becomes hot.
      (3) Cool solution.
      (4) lter sterilize using Nalgene .45 µ filter.
      (5) Check pH; should be pH 7.2 + 0.2

   c. Storage: 4°C
   d. Shelf life: 6 months
   e. Quality control:

      (1) Sterility: Add 2.5 ml of tryptic soy broth. Incubate for 48 hours.
      (2) Performance: See "performance" section of Paraffin medium with McClung's Carbon-Free Broth.

9. Paraffin Medium with McClung's Carbon-Free Broth - for the isolation of *Nocardia* from contaminated sites

   a. Formulation: Paraffin (Tissue Prep Pellets/Fischer)
      McClung's Carbon-Free Broth
   b. Preparation:

      (1) Fill 16 x 125 mm glass screw cap tubes 60% full with paraffin pellets.
      (2) Place on slanted rack and autoclave for 15 minutes at 121°C.
      (3) Let tubes harden in slanted position; then place upright.
      (4) Perform sterility check as outlined below.
      (5) Add 2.5 ml of sterile McClung's carbon-free broth to paraffin slants; tighten caps.

   c. Storage conditions: 4°C
   d. Shelf life: 6 months
   e. Quality control:

      (1) Sterility: add 2.5 ml of tryptic soy broth to each paraffin slant selected for sterility QC. Incubate for 48 hours.
      (2) Performance: inoculate paraffin-McClung broth slant with *Nocardia asteroides*. Check for good growth.

V. **SUMMARY OF IDENTIFICATION TECHNIQUES**:

Suspected Mould	Required	Recommended
Aspergillus fumigatus		Temperature tolerance, 48°C
Blastomyces dermititidis	Exoantigen, DNA probe	
Basidiobolus sp.		Forcibly ejected sporangiola
Cladosporium carrionii	Temperature tolerance, 35°C	
Coccidioides immitis	Exoantigen, DNA probe	
Conidiobolus sp.		Forcibly ejected sporangiola
Histoplasma capsulatum	Exoantigen, DNA probe	
Microsporum audouinii	Rice grains	
Microsporum canis	Rice grains	
Mycelia Sterilia	35-37°C tolerance; cylohexamide snsitivity Subculture to a nutrient deficient medium.	
Paracoccidioides brasiliensis	Mould-yeast conversion	
Rhizomucor pusillus		Temperature tolerance, 48-55°C
Sepedonium sp.	Morphology and negative Exoantigen or DNA for *Histoplasma*	
Sporobolomyces sp.		Forcibly ejected conidia
Sporothrix schenckii	Mould-yeast conversion	
Trichophyton concentricum	Trichophyton agase 1,2,3,4	
T. Equinum	Trichophyton agars 1, 5	
T. megninii	Trichophyton agars 6, 7	
T. mentagrophytes		*In vitro* hair perforation Urea hydrolysis
T. rubrum		*In vitro* hair perforation Urea hydrolysis
T. tonsurans	Trichophyton agars 1,2,3,4	
T. verrucosum	Trichophyton agars 1,2,3,4 Grown enhancement at 37°C	
T. violaccum	Trichophyton agars 1,2,3,4	
T. gallinae	Trichophyton agars 6, 7	
Wangiella dermatitidis		Temperature tolerance, 40°C
Xylohypha bantiana		Temperature tolerance, 42°C

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