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Issued by: LABORATORY MANAGER	Original Date: March 22, 2001	
Approved by: Laboratory Director	Revision Date: October 19, 2004	
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MYCOLOGY MANUAL

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LAB SAFETY

Refer to [Laboratory Safety Manual](#)

Note the following when processing Mycology isolates:

ALL work on filamentous fungus is carried out in LAMINAR AIRFLOW BIOSAFETY CABINET TYPE 2. Bio safety Level 2 procedures are recommended for personnel working with clinical specimens that may contain dimorphic fungi as well as other potential pathogenic fungi. Gloves should be worn for processing specimens and cultures.

If the **FILAMENTOUS FUNGUS FORM** of a dimorphic fungus is growing or suspected, **BIOSAFETY LEVEL 3** procedure and containment should be followed i.e. wear a N95 mask in addition to what is required for Bio safety Level 2 containment.

Wipe off working area with freshly prepared phenolic compound solution before and after each day's work. If a culture is dropped or spilled, pour a freshly prepared 1% sodium hypochlorite over the contaminated area, cover with paper towels and let stand for at least 15 minutes. Wipe off the surface and deposit the contaminated material in an appropriate biohazard disposal container. Clean the surface again using 70% alcohol.

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DAILY ROUTINE OF THE MYCOLOGY LAB

1. Check and record the temperature readings of all incubators, refrigerators, and freezers every morning. If there is an abnormal reading, report it to the charge technologist.
2. New cultures received are sorted, matched with the daily worklist, placed in numerical order (accession #) and separated according to reading schedule, and length of incubation. Fungal culture plates are examined, sealed with Parafilm and then placed in appropriate stacks and incubated at 28°C. Any culture medium showing fungal growth is removed for further work-up.
3. Fungal smears are stained and read twice daily - once before noon and again in the afternoon. All smears must be read within 24 hours except on Weekends and Holidays. All positive smears showing septate/aseptate hyphae, *Pneumocystis carinii* or yeast suggestive of Histoplasma, Blastomyces or Cryptococcus are checked by the senior mycology technologist or the microbiologist.
4. **Screening and reading cultures:**
 - i) CSF, blood and lung biopsy (and special request) cultures are read daily for the first two weeks and two times a week for the remaining incubation period. Positive specimens are worked up immediately.
 - ii) Routine screening of all other fungal cultures is done three times a week for the first two weeks and two times a week for the remaining incubation period. LPAB (Lactophenol Aniline Blue) preparations are made at least twice a week or daily depending on volumes. Any mold referred from the bacteriology section is processed and worked up the same day (except weekends and holidays). All LPAB preparations are checked by the senior mycology technologist or the microbiologist.

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SPECIMEN COLLECTION AND TRANSPORTATION

See [Pre-analytical Procedure - Specimen Collection QPCMI02001](#)

PROCESSING OF SPECIMENS

See [Specimen Processing Procedure QPCMI06003](#)

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ISOLATION AND IDENTIFICATION

I. Reading of cultures

Specimen	Incubation Period (Weeks) at 28°C	Comment
Special Request Dimorphic	6 weeks	Read daily for 2 weeks; then 3 times per week for the remaining 4 weeks.
Isolator Blood Cultures Tissues Sterile Fluids Respiratory Tract Specimens	4 weeks	Read daily for 1 week; then 3 times per week for the remaining 3 weeks.
BAL (Routine Lung Transplant)	2 weeks	Read daily for 1 week; then 3 times per week for the remaining 1 week.
Environmental	5 days	Read on Day 1 and then on Day 5.
Special Request Malassezia	1 week	Read daily for 1 week
Other specimens	3 weeks	Read daily for 1 week; then 3 times per week for the remaining 2 weeks.

II. Identification

A) FILAMENTOUS FUNGI

Introduction:

Most filamentous fungi can be identified based on a combination of colonial morphology and microscopic features. Pathogenic dimorphic fungi such as *Blastomyces*, *Histoplasma*, *Sporothrix*, etc., can often be presumptively identified by the presence of their characteristic conidia seen on Lactophenol Aniline Blue (LPAB) preparations of culture isolates.

The extent to which a filamentous fungus is identified in the laboratory will depend on several factors.

The following should be used as a guide. If there is any question regarding the extent to which a filamentous fungus should be identified, consult with the microbiologist or senior mycology technologist.

a) Sterile site specimens:

Identify all filamentous fungi isolated. Possible culture contaminants (e.g. a single colony of *Penicillium* species or other saprophytes growing on only one of several

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media) should be checked with the Senior Technologist or the Microbiologist before proceeding.

- b) All other specimens:
Identify all filamentous fungi isolated.

Procedure:

Examine the culture plates as per [Reading of Cultures Schedule](#) and record the macroscopic and microscopic findings in the LIS Media Comment field.

Macroscopic Examination

1. Colonial morphology
2. Surface pigment on non-blood containing medium
3. Reverse pigment on non-blood containing medium
4. Growth on cycloheximide containing medium

Microscopic Examination

1. Prepare a tease mount or scotch tape preparation of each fungus colony type from each media using Lactophenol Aniline Blue (LPAB).
2. Under the light microscope, examine the slide(s) for the presence, shape, size and attachment of conidia. Compare and match the above features with those described in a reference textbook.
3. If the filamentous fungus can be identified from the LPAB preparation, mark the identified colony (ies) with an “X” on the back of the culture plate(s) [if more than one type of fungus is identified, place number (e.g. 1, 2, 3, etc) beside the “X” which matches the number and identification entered into the LIS]. Re-incubate the original culture plates for the remaining incubation period and examine plates for additional growth.

Report the identification according the instructions in the [Reporting Section](#).

4. If the filamentous fungus is producing conidia but cannot be identified, determine the significance of the isolate whether it is a probable pathogen, a possible pathogen (i.e. opportunistic fungus) or an unlikely pathogen (i.e. saprophyte), take into consideration the following:
 - Direct smear result
 - Pathology report if available
 - Clinical data
 - Growth on cycloheximide containing media
 - Growth at 37°C

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- Refer to the Identification Flow Charts.
See the Senior Technologist or Microbiologist for consultation if needed.
5. Set up a slide culture (see **Appendix VI - Slide Culture**) if full identification is needed.
 6. If the filamentous fungus does not produce conidia, subculture the fungus onto the media as outlined below. Re-incubate the original plates for the remaining incubation period.

Media	Incubation	
Coloured Mould:		
Potato Dextrose Agar (PDA)	O ₂ ,	28°C
SAB	O ₂ ,	37°C

White Mould:		
Potato Dextrose Agar (PDA)	O ₂ ,	28°C
Mycosel Agar	O ₂ ,	28°C
SAB	O ₂ ,	37°C

- i) Examine the sub-cultured plates daily and record findings in the LIS Media Comment field.
- ii) If there is no growth after 7 days, forward the original culture plate to the Public Health Laboratory (PHL) for further work-up.
- iii) When sufficient growth is noted, record:

Macroscopic Examination:

- a) Colonial morphology
- b) Surface pigment
- c) Reverse pigment
- d) Growth on cycloheximide containing agar

Microscopic Examination:

- a) Prepare LPAB preparation(s) from subculture plates as required depending on colonial morphology on each plate and examine under light microscope as outlined above.
- b) If there is growth without conidia production and growth on SAB 37°C plate, send the isolate to PHL for further work-up.
- c) If there is growth without conidia production and no growth on SAB 37°C plate, determine the significance of the isolate by taking into consideration the following:

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- Direct smear result
- Pathology report if available
- Clinical data
- Growth on cycloheximide containing media
- Growth at 37°C
- Refer to the Identification Flow Charts.

See the Senior Technologist or Microbiologist for consultation if needed.
Send the isolate to PHL for further work-up if needed.

- d) If the isolate cannot be identified by slide culture, send the isolate to PHL for further work-up.
- e) If there is growth with conidia and the isolate cannot be identified, set up a slide culture (see [Appendix VI - Slide Culture](#)). If the isolate cannot be identified by slide culture, send the isolate to PHL for further work-up.

B) YEAST

If yeast is isolated from fungal media, check the bacteriology culture results. If yeast has already been identified in bacteriology, do not repeat the identification, but simply refer to the bacteriology result.

If yeast is isolated from fungal media and not in bacteriology media, identify yeast as follows:

- 1) Sterile sites and biopsy specimens:
 - a) Germ tube: **Positive** - Report as "*Candida albicans*" "isolated".
 - b) Germ tube: **Negative** - Set up: Cornmeal Agar at 28°C
SAB at 28°C
API 20C at 28°C
- 2) Respiratory sites isolates:
Check Bacteriology culture media to determine the amount of commensal flora. Then determine the significance and work-up of the yeast grown on fungal media as follows:
Significant growth – For sputum ($\geq 2+$ growth OR 1+ growth and predominant and if pus cells are seen on gram stain) OR for bronchoscopy specimen (amount greater than that of commensal flora):
 - a) Germ tube: **Positive** - Report as "*Candida albicans*"

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- b) Germ tube: **Negative** - Rule out *Cryptococcus* using Urease test. If Urease is negative, report as "Yeast, not *Candida albicans* or *Cryptococcus*". If Urease is positive, confirm purity and set up:
- BA at 37°C
 - Cornmeal Agar at 28°C
 - SAB at 28°C
 - API 20C at 28°C
 - EBM at 28°C (if it was not on original EBM)

Insignificant growth – i.e. any amount of yeast other than what has defined as significant growth.

Rule out *Cryptococcus* using Urease test. If Urease is negative, report as part of Commensal flora **without** specifically mentioning the presence of yeast. If Urease is positive, confirm purity and set up:

- BA at 37°C
- Cornmeal Agar at 28°C
- SAB at 28°C
- API 20C at 28°C
- EBM at 28°C (if it was not on original EBM)

- 3) Voided urines, superficial sites, wounds and drainage fluids:
No Germ tube performed. Report as "Yeast" with quantitation. No further work-up is required.
- 4) Isolates from all other sites:
- a) Germ tube: **Positive** - Report as "*Candida albicans*".
 - b) Germ tube: **Negative** - Report as "Yeast, not *Candida albicans*".

If yeast is referred to **Mycology from bacteriology media (i.e. Germ tube – positive)**, identify yeast as follows:

- 1) Sterile sites and biopsy specimens:
Set up: Cornmeal Agar at 28°C
SAB at 28°C
API 20C at 28°C
- 2) Respiratory sites isolates (Germ tube – Positive and Urease – Positive):
Set up: BA at 37°C
Cornmeal Agar at 28°C
SAB at 28°C
API 20C at 28°C
Urease at 28°C (repeat)

Refer to Yeast Identification Flow Chart for Identification.

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The acceptance of API 20 C is >90% and must agree with cornmeal result. Refer unidentifiable isolates to the PHL for further work-up.

C) NOCARDIA

Nocardia species are aerobic members of the actinomycetes which are gram positive branching filamentous bacilli that fragment into rod-shaped to coccoid elements. Most clinical infections are due to *N. asteroides* and *N. brasiliensis*. Most specimens from patients with suspected Nocardiosis will be respiratory specimens (e.g. sputum, BAL, lung biopsy, etc.) although tissue (eg. Mycetoma) and body fluid may also be submitted. For identification, proceed as follows:

1. When Nocardia isolation is requested or organisms suggestive of Nocardia are seen on gram stain, plant specimen onto Sodium Pyruvate Agar (PYRA) and incubate in O₂, 28°C for 4 weeks in Mycology. As well, the Blood Agar (BA) and Chocolate (CHOC) plates should be kept in bacteriology and incubated in O₂, 35°C for 48 hours and then send it to Mycology for the rest of the 4 weeks. Send plates to mycology for further work up. See Table 1 for nocardia work up.

Table 1. Differentiation of *Nocardia*, *Streptomyces*, Atypical Mycobacteria based on colonial and microscopic features.

	<i>Nocardia</i> Species	<i>Streptomyces</i> species	Atypical Mycobacteria
Gram Stain	GPB*	GPB	GPB
Modified Kinyoun stain	Partially Acid-fast	-	+
Regular Kinyoun stain	-	-	+
Morphology	Filamentous, Branching	Filamentous, Branching	Bacillary
MacConkey (without Crystal Violet)	No Growth	-	Growth
Strong musty smell	-	+	-
Adherence to agar	+	+	-
Report as:	Phrase 1	Phrase 2	Phrase 3

*GPB = Gram positive bacilli

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REPORTING

1. FUNGAL STAIN:

Negative Reports: "No Fungal elements seen".

NB: In LIS, status these reports as final. No verification required.

Positive Reports: as per keypad options:

- Yeast seen (with quantitation)
- Yeast with pseudohyphae seen (with quantitation)
- Filamentous fungus seen (without quantitation); with morphologic description of organisms/structures seen (e.g. septate hyphae). Define structures.

If unable to interpret fungal elements, consult Senior Mycology Technologist.

NB: In LIS, status these reports as final. Positive smears with rare and unusual structures (fungal elements) must be checked by Senior Mycology Technologist.

2. CULTURE:

Negative Reports: "No Fungus isolated".

NB: In LIS, status these reports as final. No verification required.

Positive Reports:

- When reporting a fungus culture result, DO NOT quantitate filamentous fungi.
- If fungus has already been identified and **reported under bacteriology result**, then enter one of the following phrases as appropriate in the **Test Comment Field**:
 - a) "Please see Culture and Sensitivity report for fungus isolate(s)". (Use this phrase when no additional fungi are isolated on the fungal media).
 - b) "Please see Culture and Sensitivity report for additional fungus isolate(s)". (Use this phrase when additional fungi are isolated on the fungal media)

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Add the appropriate phrases:

- a) "(Organism name)"
- b) "(Organism name); normally non-pathogenic".
- c) "(Organism name or description); cannot rule out contamination".
- d) "Non-sporulating fungus, normally non-pathogenic".
- e) "Filamentous fungus; further identification to follow".
- f) "(Organism name); Confirmation to follow".

NB: In the LIS, status the report as interim (^L).

- For organisms **isolated in fungal media only:**

Yeast:

All sites except sterile sites and BAL, report yeast with quantitation

For sputum,

Significant growth: *Candida albicans*

“Organism name”

Cryptococcus neoformans

Insignificant growth: “Yeast isolate; normally commensal flora”

Filamentous Fungi:

If the filamentous fungus is deemed to be **significant**, and when the work-up and identification of the isolate is complete, use one of the following phrases as appropriate:

- a) "(Organism name)"
- b) "(Organism name); normally non-pathogenic".
- c) "(Organism name or description); cannot rule out contamination".
- d) "Non-sporulating fungus, normally non-pathogenic".
- e) "Filamentous fungus; further identification to follow".
- f) "(Organism name); Confirmation to follow".

NB: In LIS, status the report as interim (if further incubation required) **or** final (if no further incubation required).

If the filamentous fungus is deemed to be **insignificant**:

“(Organism name); likely not significant”.

- When a fungus isolate has been forwarded to the Public Health Laboratory (or other reference laboratory) for further identification or confirmation, use one of the following phrases as appropriate:

- a) "Filamentous fungus; further identification to follow".
- b) "(Organism name); Confirmation to follow".

NB: In LIS, status the report as interim (^L).

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- When reporting a fungus culture result, please note the following:

When the Public Health Laboratory (PHL) report becomes available, proceed as follows:

- i) Replace the isolate name or description in the **Isolate Field** with the PHL result.
 - ii) In the **Isolate Comment Field**, remove the statement "Further identification to follow" or "Confirmation to follow" and enter the following phrase (from the keypad) and add the PHL report number: "Public Health Laboratory Report No. _____".
 - iii) Status the report as final (^F).
- ***Nocardia***
 - a) "*Nocardia* species; further identification to follow".
 - b) "Branching Gram positive bacilli; further identification to follow".
 - c) "Mycobacterium species isolated."
 - d) Forward isolates to the Public Health Laboratory (PHL) for further identification.

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ACID-FAST STAIN FOR NOCARDIA (MODIFIED KINYOUN)

Principle

Nocardia species possess the unique characteristic of resisting decolorization with acid alcohol.

Reagents

1. Carbol-fuchsin

Basic fuchsin solution	(3 g basic fuchsin in 100 mL 95% ethyl alcohol)
	10 mL
Phenol 5% aqueous	90 mL

2. Decolourizer (1% sulfuric acid)

H ₂ SO ₄ (concentrated)	1 mL
Distilled water	99 mL

3. Methylene blue

Methylene blue	0.3 g
Distilled water	100 mL

Staining Procedure

1. Fix the smear by gentle heating.
2. Flood the smear with Carbol fuchsin solution.
3. Allow the slide to stand for 5 minutes.
4. Wash the smear with tap water.
5. Decolorize the smear with 1% sulfuric acid until no more colour appears in the washing (approx. 1 min.).
6. Rinse with tap water.
7. Counterstain with methylene blue for approximately 1 minute.
8. Rinse with tap water and air dry.

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Interpretation

The filaments of *Nocardia* species and *Rhodococcus* appear red-stained against a blue background.

Quality Control

A positive control slide of *Nocardia* species is stained simultaneously with the clinical specimens.

References

1. Murray, PA. et al. Manual of Clinical Microbiology, 7th edition, 1999 ASM Press, Washington, D.C.

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CALCOFLUOR WHITE STAIN

Purpose

Calcofluor White stain is useful for staining skin scrapings, hair, nail and thick tissue specimens. Calcofluor staining requires the addition of KOH which helps to dissolve keratinized particles and helps to emulsify solid, viscous material that may mask the fungal elements. Calcofluor method is **NOT** suitable for the detection of *Pneumocystis carinii*. Calcofluor stained smears are read under the UV microscope as for the Fungi-Fluor™ stain.

Procedure

1. Place a portion of the specimen on the slide (select a purulent area if secretions).
2. Add 1 to 2 drops KOH and emulsify specimen. If it does not clear rapidly, place slide in petri dish and allow to stand about 10 minutes.
3. If tissue or scrapings, place the slide on 35⁰C bench top heating block for 15-20 minutes to speed clearing.
4. Add 1 or 2 drops of calcofluor white reagent and mix thoroughly. Calcofluor white may be added right after KOH.
5. Apply coverslip gently. Make sure specimen does not overflow.
6. Examine under fluorescent microscope (see Fungi-Fluor™ stain).
7. Always include a control slide positive for yeast or filamentous fungus.

NB: In the event when no Fungal Stain smear is available, Gram smear may be retrieved for over staining by Calcofluor White or KOH.

Interpretation

Fungal cell walls fluoresce apple green (see Fungi-Fluor™ stain).

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References

1. Manufacturers' Instructions:
Calcofluor White Reagent - Difco.
2. Clin. Micro. Newsletter 9:33-36, Mar. 1, 1987. K.L. McGowan. "Practical Approaches to Diagnosing Fungal Infections in Immunocompromised Patients".

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FUNGI-FLUOR™ STAIN

Purpose

The Fungi-Fluor™ stain is used for the rapid identification of various fungal elements in fresh or frozen clinical specimens.

Principle

The active, colourless, fluorescing dye in the staining solution is Cellufluor which is the disodium salt of 4,4'-bis[4-anilino-6-bis-(2-hydroxyethyl) amino-s-triazin-2-ylamino]-2,2'-stilbenedisulfonic acid. Fungi-Fluor™ staining solution is a 0.05% solution of this dye in deionized water with potassium hydroxide added as a clearing agent. The Fungi-Fluor™ counter staining solution B is an aqueous solution of Evans Blue dye used to reduce background fluorescence. Cellufluor binds nonspecifically to beta-linked polysaccharides found in chitin and cellulose which are present in fungal cell walls.

When exposed to long wave UV light, fungal cell walls will fluoresce.

NB: Collagen, elastin, cotton fibres, plant material, some cells, cell inclusions and parasite cyst forms (eg. Acanthamoeba) may fluoresce.

Materials

Staining Solution A

Counterstaining Solution B

Absolute alcohol

Water

Fluorescent Microscope (Leitz Ortholux with G filter module exciting filter BP 350-460, suppression filter LP515 or equivalent)

Precautions

1. Store in a dark or opaque bottle, tightly sealed, at room temperature.
2. Avoid eye or skin contact: use gloves and protective glasses.

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Procedure

1. Prepare smear of specimen and allow to air dry.
2. Fix on the rack with absolute methanol for 5 minutes until dry. Fixed smears can be held indefinitely until ready to stain and examine.
3. Add a few drops of Fungi-Fluor solution A (Cellufluor) for 1 minute.
4. Rinse gently with tap water.
5. Apply coverslip to wetted slide and examine with the fluorescent microscope using the designated filter. If there is a delay, add fresh distilled water to the coverslip just prior to examination.
6. Optional for thicker smears. Add few drops of the counterstain Fungi-Fluor solution B. Rinse gently with tap water and then proceed as in step 5 above.

NB: Gram stained smears can be overstained with Fungi-Fluor after removing immersion oil with alcohol. Similarly, Fung-Fluor stained slides may be overstained with other stains such as GMS, PAS, Geimsa, etc.

Quality Control

Stain a smear of *Candida albicans* daily.

Interpretation

Use 25x or 40x objective.

Fungal cell walls will fluoresce apple-green. Observe for characteristic morphology to differentiate from artifacts and background. When the counterstain is used, fungi will appear yellow-green against a red-orange background.

Appearance of other structures / organisms:

- i) Fungal elements - intense peripheral staining with characteristic morphology.
- ii) *Pneumocystis carinii* - fainter staining cyst wall (5-7 µm diameter) and intensely staining internal "bean-shaped" or "double-parenthesis-like" structures with apposed sides flattened.

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- iii) *Acanthamoeba* sp. cysts - intensely staining double wall with wrinkled outer wall
(10-25 µm diameter)

References

1. Manufacturers' Instructions (Data Sheet #316). Fungi-Fluor™ kit - Polysciences, Inc., July 1995
2. V.S. Baselski et al. "Rapid Detection of *Pneumocystis carinii* in Bronchoalveolar Lavage Samples by Using Cellufluor Staining". J. Clin. Micro. 28:393-394, Feb. 1990.
3. D. H. Larone. Medically Important Fungi. A Guide to Identification. 3rd ed. 1995, ASM Press.

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Section: Mycology Bench Manual	Subject Title: GMS and PAS Fungal Stains	
Issued by: LABORATORY MANAGER	Original Date: March 22, 2001	
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Gomori Methenamine Silver (GMS), Periodic Acid Schiff (PAS) and Fontana Masson FUNGAL STAINS

Purpose:

These are special fungal stains, usually ordered by the Microbiologist when further identification of fungi is required.

1. Fix smears in absolute methanol for 5 minutes.
2. Fill out yellow Histopathology requisition form.
3. Label slides with specimen number, name of stain and "Micro".
4. Send to Histopathology for staining.
5. Stained slides will be returned to the Mycology section for interpretation. These must be reviewed by a senior technologist in mycology or by the microbiologist.

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Section: Mycology Bench Manual	Subject Title: India Ink	
Issued by: LABORATORY MANAGER	Original Date: March 22, 2001	
Approved by: Laboratory Director	Revision Date:	

INDIA INK

Purpose

The procedure is applicable only to suspected positive Cryptococcal cultures.

Procedure

1. For suspected Cryptococcal cultures, make a wet preparation using saline on a clean glass slide, then add a small drop of India Ink and mix.
2. Apply a large coverslip ((22 x 40 mm) over the mixture and press it gently to obtain a thin mount.
3. If India Ink is too thick (dark), dilute it by 50% with saline.
4. Allow the preparation to stand for few minutes to settle.
5. Scan under low power in reduced light. Switch to high power if necessary.

Interpretation

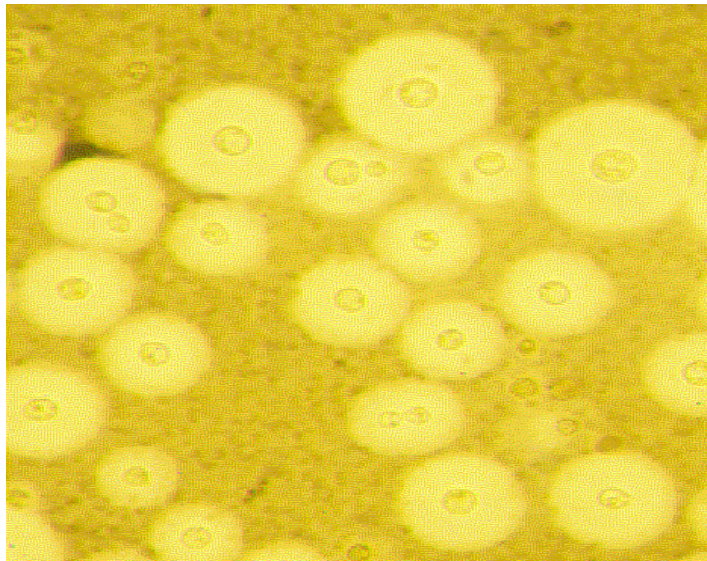
The mucoid capsule appears as a clear halo that surrounds the yeast cell or lies between the cell wall and the surrounding black mass of India Ink particles. Capsules may be broad or narrow. The yeast cells may be round, oval or elongate. Buds may be absent, single or rarely multiple and may be detached from the mother cell but enclosed in a common capsule attached.

Reference

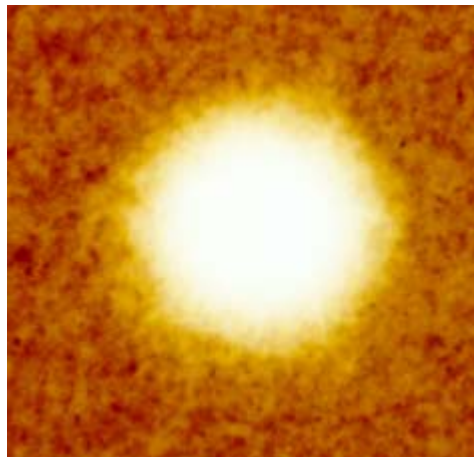
Murray PA, et al. Manual of Clinical Microbiology 7th ed., 1999, ASM Press.

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India Ink positive for Cryptococcus



Artefacts produced by reaction of India Ink (False Positive)



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Section: Mycology Bench Manual	Subject Title: Lacto Phenol Aniline Blue (LPAB)	
Issued by: LABORATORY MANAGER	Original Date: March 22, 2001	
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LACTOPHENOL ANILINE BLUE (LPAB)

Purpose

To determine the morphology of the conidiogenous cells and the conidia that they give rise to in order to identify a filamentous fungus.

Principle

LPAB contains lactic acid as a clearing agent, phenol as a disinfectant, glycerol to prevent drying and Aniline Blue which is the dye that stains fungi. LPAB is a wet preparation.

Procedure

1) TEASE PREP

The test must be performed in the Laminar Airflow Biosafety Cabinet. First, observe the gross morphology of the colony carefully to determine whether or not the culture is mouldy, granular or a mixture of both. It is important to prepare the LPAB preparation by "teasing" the fungus not "chopping" it.

Materials required for LPAB staining:

1. LPAB reagent
2. Probe to get the specimen
3. Teasing needles
4. Glass slides
5. Coverslips
6. Lead or wax pencils
7. Disinfectant bucket
8. Electric incinerator
9. Clear nail polish
10. Slide tray

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Tease Prep Procedure:

1. Sterilize the loop and the needles in the incinerator and allow them to cool.
2. Label slide, place 2 drops of LPAB reagent on the slide.
3. Cut a small piece of the fungus from a granular or colored part of the colony, somewhere away from the central part towards the periphery and place the piece of the fungus in the LPAB in the upside down position.
4. Hold the thallus with the needle and gently tease the inverted side of the specimen into the staining (LPAB) fluid.
5. After enough teasing, remove all the solid particles and the agar from the mixture and discard in the disinfectant container.
6. Put a coverslip gently onto the LPAB preparation and hold the slide preparation briefly over the incinerator opening. Heating the slide will help to stain the cell wall of the fungi and kill the spores on the surface of the slide.
7. Seal the preparation with nail polish if necessary for a permanent mount.
8. Examine under the light microscope using the low power objective.

2) SCOTCH TAPE PREP FROM PLATE CULTURE (Not done on suspected dimorphic fungi)

1. Place a drop of LPAB onto a clean glass slide.
2. Take a small piece of clear scotch tape and loop back on itself with sticky sides out.
3. Hold the tip of the loop securely with forceps.
4. Press the sticky side firmly to the surface of the fungal colony.
5. Pull the tape gently away from the colony.
6. Open up the tape strip and place it on the drop of LPAB on the glass slide, making sure that the entire sticky side adheres to the slide.
7. Examine under the light microscope.

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Reference

Davise H. Larone. Medically Important Fungi. A Guide to Identification. 3rd ed. 1995, ASM Press.

Martha E. Kern. Medical Mycology. 1985.

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Section: Mycology Bench Manual	Subject Title: Isolator 10 Blood Culture System for Dimorphic Fungi	
Issued by: LABORATORY MANAGER	Original Date: March 22, 2001	
Approved by: Laboratory Director	Revision Date:	

ISOLATOR 10 BLOOD CULTURE SYSTEM FOR DIMORPHIC FUNGI

I. Introduction

The Isolator 10 blood culture system should be used for the isolation and detection of dimorphic fungi such as Histoplasma and Blastomyces.

If BacT/Alert bottles are received with a request for dimorphic fungi, notify the ward / ordering physician that they must use the Isolator 10 collection tubes. The BacT/Alert bottles should only be processed as per routine blood cultures.

II. Collection and Transport

Using aseptic technique, collect 10 ml of blood into a clean, sterile syringe. Transfer the blood into an Isolator 10 microbial tube. Transport to the laboratory immediately for processing. If a delay in transport or processing is anticipated, the tubes can be held for 24 hours at room temperature.

III. Reagents / Materials / Media

Refer to Appendix I.

IV. Procedure

A. Processing of Isolator 10 Microbial Tubes:

1. Centrifuge specimen at 4000 rpm for 30 minutes.
2. Disinfect the stopper using 10% PVP iodine or tincture of iodine. Allow to dry completely.
3. Place cap over stopper. Grasp only the sides of the cap.
4. Position cap under press and pull down handle and release.
5. Collapse bulb of supernatant pipette completely before inserting stem into the tube.

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Blood Culture Manual		

6. Insert stem into tube and release bulb to withdraw supernatant fluid. Discard the supernatant.
NB: The use of a safety hood is mandatory for steps 6 to 9.
7. Vortex the tube for at least 10 seconds at the highest setting.
8. Collapse bulb of concentrate pipette completely and then insert stem into tube. Slowly withdraw all concentrate.
9. Dispense concentrate in a straight line along the surface of the agar. Keep inoculum away from the edge of the plate.

Media	Incubation
	O ₂ at 28°C
Inhibitory Mold Agar (IMA)	x 4 weeks
Esculin Base Medium (EBM)	x 4 weeks
Blood Egg Albumin Agar (BEAA)	x 4 weeks
Blood Egg Albumin Agar (BEAA)	x 4 weeks

10. Using the tip of the pipette, streak the plates. Use 15-20 passes perpendicular to the original inoculum line.
11. Forward plates to Mycology for incubation and processing.

B. Interpretation of Fungal Culture Plates:

Refer to Mycology Manual.

V. **Reporting Results**

Refer to Mycology Manual.

VI. **Reference**

1. Isolator 10 Product Insert.

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Julius Kane, Richard Summerbell, Lynne Sigler, Sigmund Kraiden and Geoffrey Land. Laboratory Handbook of DERMATOPHYTES. A Clinical Guide and Laboratory Manual of Dermatophytes and other Filamentous Fungi from Skin, Hair and Nails. 1997, Star Publication.

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E.W. Koneman, Glenn D. Roberts. Practical Laboratory Mycology. Third Edition, 1985, Williams & Wilkins.

J.W. Rippon. Medical Mycology. The Pathogenic Fungi and the Pathogenic Actinomycetes. Third Edition, 1988, W.B. Saunders.

E.W. Koneman et al. Color Atlas and Textbook of Diagnostic Microbiology. 5th Edition, 1997, J.B. Lippincott.

G.J. Hageage Jr. et al. Use of Calcofluor White in Clinical Mycology. Laboratory Medicine 15:109-112, 1984.

J. Barenfanger. Identification of Yeasts and Other Fungi from Direct Microscopic Examination of Clinical Specimens. Clin. Micro. Newsletter 12:9-15, Jan. 15, 1990.

Davis H. Larone. The Identification of Dematiaceous Fungi. Clin. Micro. Newsletter 11:145-150, Oct. 1, 1989.

K.J. Kwon-Chung, J.E. Bennett. Medical Mycology. 1992, Lea & Febiger.

Bailey & Scott's Diagnostic Microbiology. Eleventh Edition by Betty A. Forbes, Daniel F. Sahn & Alice S. Weissfeld. 2002 (Mosby).

Medically Important Fungi. A Guide to Identification, 4th. Edition by Davise H. Larone. 2002 (ASM Press).

Topley & Wilson's Microbial Infections, ninth edition, volume 4, medical mycology by Leslie Collier, Albert Balows and Max Sussman, 1998 (Arnold).

Atlas of Clinical Fungi, 2nd. Edition by Dr. G.S. de Hoog, J. Guarro, J. Gene & M.J. Figueras. CBS Utrecht, the Netherlands 2000.

www.doctorfungus.com

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Section: Mycology Bench Manual	Subject Title: Appendix I - Conversion	
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APPENDIX I

CONVERSION (Converting Mycelial Phase of Dimorphic Mould to a Yeast Phase)

I. Purpose

To be used only for suspected *Sporothrix schenckii* and *Blastomyces dermatitidis*. Send all other suspected dimorphic fungi to the Public Health Laboratory for identification and/or confirmation.

II. Procedure

1. Transfer a large inoculum of the filamentous culture onto the surface of a fresh, moist slant of Brain Heart Infusion Agar (BHIA). If the slant is dry, add 0.5 ml sterile distilled water before inoculation.
2. Incubate culture at 37⁰C.
3. Examine daily for the presence of yeast-like, creamy colonies.

NB: It is not necessary for the whole culture to convert. *S. schenckii* usually converts rapidly to the yeast phase without the need for further 37⁰C subcultures.

4. Confirm the presence of any yeast forms by LPAB tease prep (Refer to Lactophenol Analine Blue (LPAB) procedure in the Staining Methods).

III. Reference

Koneman, E.W., et al. 1997. Color Atlas and Textbook of Diagnostic Microbiology, 5th edition. J.B. Lippincott.

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Section: Mycology Bench Manual	Subject Title: Appendix II - Cornmeal Tween-80/OXGALL	
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APPENDIX II

CORNMEAL TWEEN-80/OXGALL AGAR

I. Purpose

To be used for yeast morphology when the germ tube is negative, but further identification is required as outlined in the section: Isolation and Identification. Cornmeal Tween-80 provides excellent diagnostic morphological features for yeast identification, but produces chlamydospores more slowly than oxgall agar.

II. Procedure

A. Cornmeal Tween 80:

1. Using a sterile wire loop, inoculate a small portion of a yeast colony by making two parallel streaks a few mm apart on the surface of a cornmeal agar plate. Do not cut into the agar.
2. Streak over the lines in a "zigzag" fashion (Dolmau technique).
3. Place a clean coverslip over the streaked area and press gently.
4. Include controls *T. glabrata* (negative) and *C. parapsilosis* (positive).
5. Incubate at 25-28⁰C for 48 hours.
6. Using the light microscope examine under low power and high dry objectives for the presence of hyphae, pseudohyphae, blastoconidia, chlamydospore and/ or arthroconidia. The plate may need to be reincubated if the morphology is not fully developed (eg. arthroconidia formation).

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B. Oxgall:

Oxgall agar is specifically used to show chlamyospores. Oxgall does not always give the classic diagnostic morphology of yeast as is seen with the Cornmeal Tween-80 agar.

1. Using a sterile spade shaped spatula, inoculate lightly by making 2 to 3 parallel cuts approximately 1/2 inch apart at 45⁰ angle. Avoid cutting the agar through to the bottom.
2. Apply a clean coverslip on the inoculated area and press gently.
3. Include controls *C. albicans* (positive) and *C. tropicalis* (negative).
4. Incubate at 28⁰C for up to 48 hours.
5. Examine the areas where the agar is cut under low and high dry objectives using the light microscope.
6. Observe for the presence of hyphae, pseudohyphae, blastoconidia, chlamyospores and arthroconidia.
7. Refer to Table 1. "Identification of yeast" in the section, Isolation and Identification for interpretation.

Note: Yeast producing chlamyospores on cornmeal and/ or oxgall are reported as *Candida albicans*. Yeast not producing chlamyospores or pseudohyphae may require further testing.

III. References

1. J.B. Fisher, Julius Kane, "Production of Chlamyospores by *Candida albicans* cultivated on dilute Oxgall Agar". Mycopath. 35:223-229, 1968.

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Section: Mycology Bench Manual	Subject Title: Appendix III - To Determine Cycloheximide Resistance of an Isolate	
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APPENDIX III

DETERMINING CYCLOHEXIMIDE RESISTANCE OF AN ISOLATE

I. Purpose

To rule out or help confirm the presence of a possible dimorphic fungus or dermatophyte. Normally used in the identification of white moulds.

The following pathogenic fungi are resistant to Cycloheximide:

Blastomyces dermatitidis
Histoplasma capsulatum
Coccidioides immitis
Sporothrix schenckii
Paracoccidioides brasiliensis
Trichophyton sp.
Microsporum sp.
Epidermophyton floccosum

Cycloheximide inhibition rules out the above fungi. Resistance to cycloheximide **may** indicate one of the above pathogens.

II. Procedure

1. Subculture the isolate to Potato Dextrose Agar (PDA) and Mycosel Agar (MYC) and incubate at 28⁰C (or RT). MYC contains cycloheximide.
2. Observe periodically for 7-10 days or until good growth on one or both media.

III. Interpretation

- i) PDA+/ MYC + : Fungus **MAY** be one of the above listed pathogens.
- ii) PDA +/- MYC - : Fungus is **NOT** one of the ABOVE listed pathogens.

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iii) PDA -/ MYC - : Test invalid, repeat.

NB: *Penicillium marneffei* (dimorphic fungus) is inhibited by cycloheximide.

IV. Reference

M.R. McGinnis. Laboratory Handbook of Medical Mycology 1980, Academic Press.

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Section: Mycology Bench Manual	Subject Title: Appendix IV - Fluorescent Microscope (Instructions)	
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APPENDIX IV

FLUORESCENT MICROSCOPE (INSTRUCTIONS)

1. Record date and time in UV Record Book when turning on the fluorescent microscope.

Note: If UV light is recently turned off, WAIT FOR ONE HOUR before turning it on again.
2. Switch on the microscope
3. Allow the microscope to warm up for 5 minutes.
4. Turn filter setting to 2 (G filter) (Leitz Ortholux Microscope) -- (right side of Grey structure above objectives. Use filter #3 for bright-field microscopy).
5. Pull out the small black ring (rod) on the left side above the revolving nose piece to let UV light pass through the objective; push it in to prevent the slide from fading when not examining the preparation.
6. When finished, turn off the "ON" button.
7. Enter the time and calculate the total time used in the UV Record Book.
8. Fluorescent bulbs are good for a maximum of 200 hours. Notify the senior technologist that the bulb should be changed when the maximum time has been reached.

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Section: Mycology Bench Manual	Subject Title: Appendix V Germ Tube Test	
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APPENDIX V

GERM TUBE TEST

I. Introduction

This is a rapid test for the presumptive identification of *C. albicans*.

II. Reagents / Materials / Media

Bovine serum - A small volume to be used as a working solution may be stored at 2 to 8°C. Stock solution can be dispensed into small tubes and stored at -20°C.

Clean glass microscope slides
Glass coverslips
Glass tubes (13 x 100 mm)
Pasteur pipettes

III. Procedure

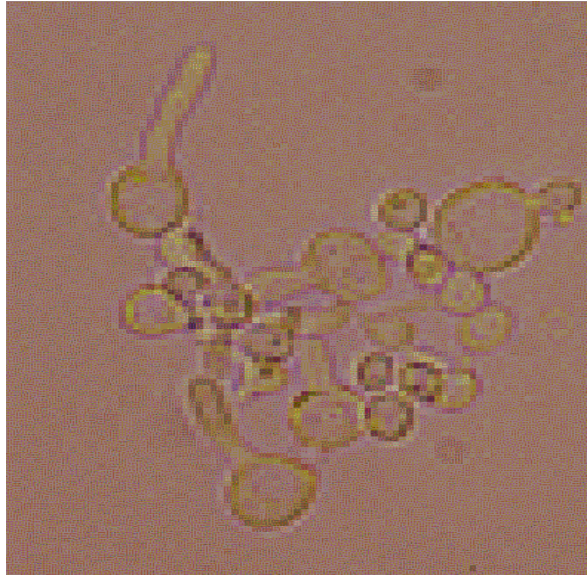
1. Put 3 drops of serum into a small glass tube.
2. Using a Pasteur pipette, touch a colony of yeast and gently emulsify it in the serum. The pipette can be left in the tube.
3. Incubate at 35⁰C to 37⁰C for up to 3 hours but no longer.
4. Transfer a drop of the serum to a slide for examination.
5. Coverslip and examine microscopically using x 40 objective.

IV. Interpretation

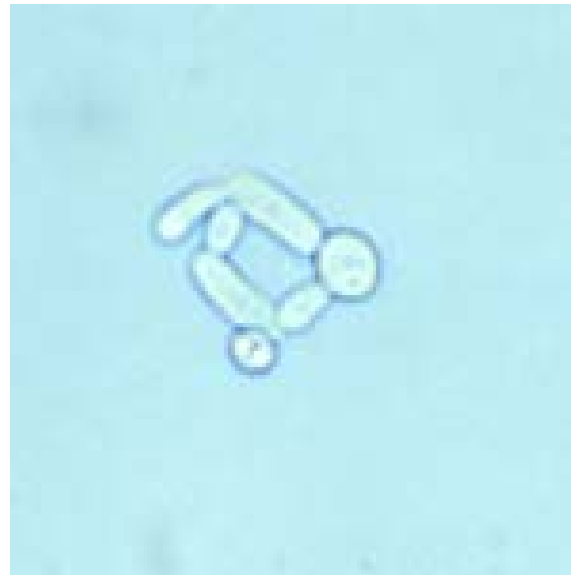
Germ tubes are appendages half the width and 3 to 4 times the length of the yeast cell from which they arise. There is no constriction between the yeast cell and the germination tube.

Positive test: presence of short lateral filaments (germ tubes) one piece structure
Negative test: yeast cells only (or with pseudohyphae) always two pieces

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Respiratory Tract Culture Manual		



Positive Germ Tube
(Parallel sides; Non-septate)



Negative Germ Tube
(Constriction at point of attachment)

Note: *C. tropicalis* may form pseudohyphae (usually after 3 hours incubation) which may be falsely interpreted as germ tube positive.

IV. Quality Control

Set up known controls daily:

- C. albicans*: positive
- C. tropicalis*: negative

V. References

1. Haley LD and Callaway CS, Laboratory Methods in Medical Mycology, CDC Publication No. 79-8361, 1979. p119.

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Section: Mycology Bench Manual	Subject Title: Appendix VI - Slide Culture	
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APPENDIX VI

SLIDE CULTURE

I. Purpose

A slide culture is used to preserve and observe the natural state and the actual structure showing conidiogenous cells and conidia of the fungus.

Before setting up a slide culture, it is important to do an LPAB preparation and demonstrate that the organism is susceptible to Cycloheximide (Exception: Dermatophytes and *Sporothrix schenckii*).

Note: NEVER SET UP A SLIDE CULTURE ON A SUSPECTED *COCCIDIOIDES*, *BLASTOMYCES*, *PENICILLIUM marneffeii* AND *HISTOPLASMA* CULTURE OR ANY WHITE MOULD THAT GROWS WELL ON MYCOSEL.

II. Procedure

1. Place a sterile filter paper in a small petri dish.
2. Add some sterile distilled water.
3. Place 2 pieces of wooden sticks on the filter paper, a few centimetres apart.
4. Place a clean alcohol flamed heat sterilised glass slide on the sticks.
5. Using a sterile spatula, cut a small square (about 1 x 1 cm) of agar block from the Potato Dextrose Agar (PDA) (or 3% Salt Agar) and place it on the centre of the slide.
6. Using a needle or spatula, inoculate the agar with a small amount of fungus under test on each of the four sides of the block.
7. Place a heat-sterilized coverslip over the block and press down gently.

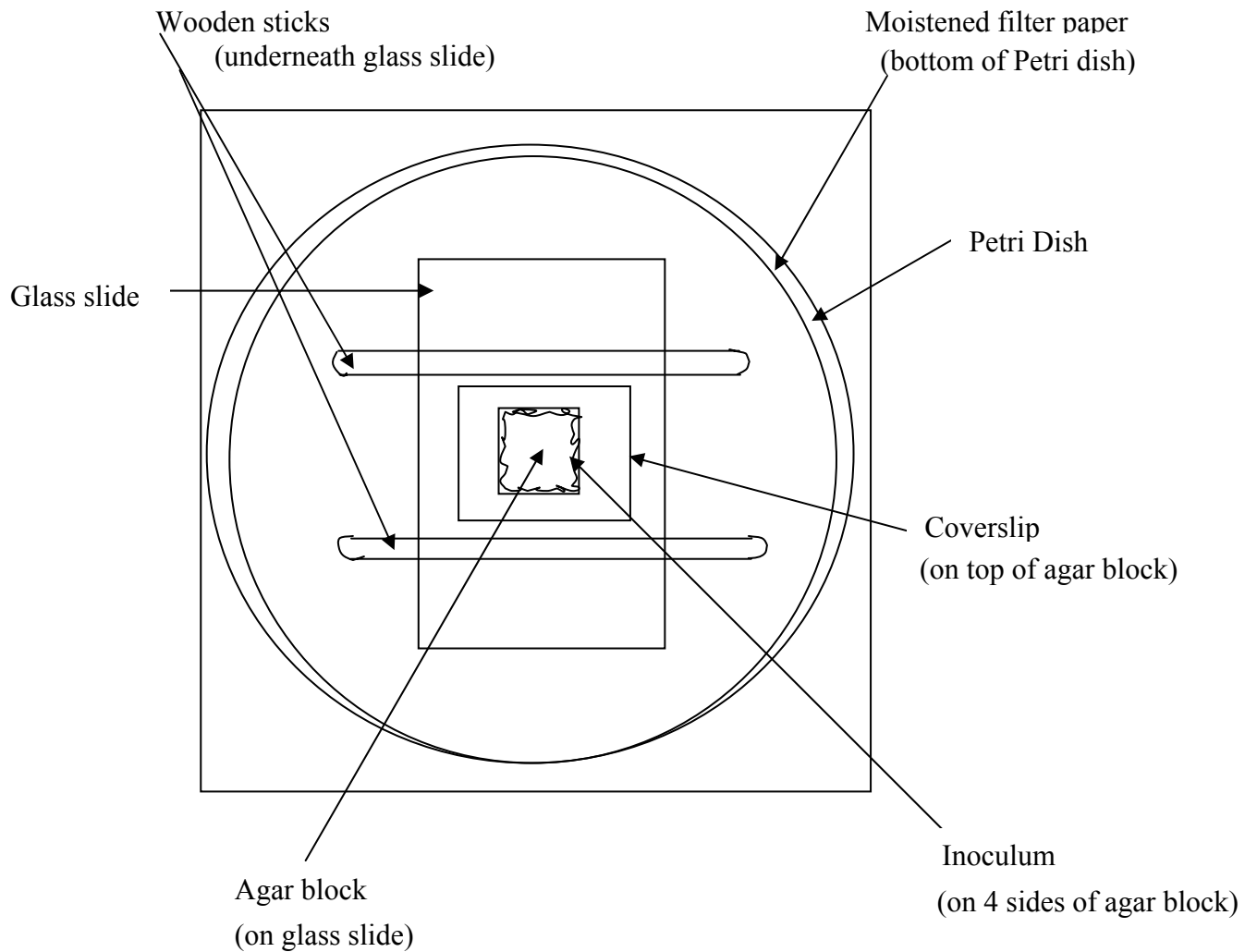
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8. Incubate at 28°C or room temperature.
9. Examine **daily** for sufficient growth.
10. When good growth appears, place a drop of LPAB on a clean slide, remove the coverslip using forceps, pass the top side of the coverslip in front of the incinerator opening to fix and place it over the LPAB on the slide.
11. Examine under the light microscope. If the preparation shows sufficiently developed structures, prepare the second preparation from the slide by removing the agar block from the slide. Discard the agar in the sharps container. Place a drop of LPAB on the slide and a new coverslip on it. Both preparations can be preserved indefinitely by sealing the edges with nail polish.
12. If the fungus is still underdeveloped, add a fresh coverslip on the agar block, reseal the plate with parafilm and continue incubation.

III. Reference

1. Davise H. Larone. Medically Important Fungi. A Guide to Identification 1995, ASM Press.

Setting up Slide Culture



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Section: Mycology Bench Manual	Subject Title: Appendix VII - Stock Cultures -Water Culture Technique	
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APPENDIX VII

STOCK CULTURES -WATER CULTURE TECHNIQUE

I. Purpose

To maintain stock cultures of fungi.

II. Procedure

1. Rub a sterile moistened swab over the surface of an actively sporulating fungal colony (or, using a spatula, scrape off the mycelial growth of the fungus above the agar).
2. Wash the swab (or spatula) off into a screw cap bijou bottle containing approximately 4 ml of sterile distilled water.
3. Tighten the cap and store at room temperature.
4. Add sterile distilled water periodically to prevent evaporation.
5. Subculture at least once a year to maintain viability of the stock cultures. Twirling the culture to resuspend the conidia, subculture using a sterile pipette.
6. Record stock cultures in the appropriate stock book in Mycology with the date the stock was made.

III. Reference

1. Davise H. Larone. Medically Important Fungi. A Guide to Identification 1995, ASM Press.

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APPENDIX VIII

I. Principle

The API 20C AUX strip consists of 20 microtubes containing dehydrated substrates in which 19 assimilation tests are performed. After inoculation and incubation, the reactions are interpreted by comparison to growth controls and use of the Identification Table provided with each kit.

II. Material

API 20C AUX Strip
Incubation tray
C Medium
Pasteur pipettes (or Plastic pipettes)
Suspension medium
RAT medium

III. Procedure

1. Create a humid atmosphere within an incubation tray by distributing 5 ml of distilled water into the bottom of the tray.
2. Use a cotton swab to suspend a portion of the yeast colony in the suspension medium equal to a 2 McFarland standard.
3. Place 1 drop of yeast suspension into RAT Medium.
4. Transfer 100 µl (3 drops) of RAT Medium suspension into an ampoule of C medium.
5. Using a pasteur pipette, fill the capsules with the suspension in C medium.
6. Put lid on and incubate at 28⁰C x 48-72 hours.
7. After 24, 48 (and 72 hrs if needed) check for growth.
8. Record results onto the supplied report form and compare results with the Identification Table to identify yeast.
9. Record results in the LIS.

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IV. Quality Control

Control strains are set up for each new lot number of strips.

Use the follow isolates:

1. *C. albicans* ATCC 14053
2. *C. guilliermondii* ATCC 6260
3. *C. pseudotropicalis* ATCC 4135

V. References

1. API 20C AUX package insert #20210.

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APPENDIX IX

MEDIA / REAGENTS

1. BLOOD EGG ALBUMIN AGAR (BEAA)

Commercially prepared by Bio-Media lab

Columbia agar base
5% sheep blood agar
Egg Albumin
Avidin
Gentamicin
Vancomycin

Purpose: Primary isolation media for fungi especially *Histoplasma capsulatum*.

Quality Control

<u>Fungus</u>	<u>Incubation Temperature</u>	<u>Results</u>
<i>Trichophyton mentagrophytes</i>	28°C	Good growth
<i>Candida albicans</i>	28°C	Inhibited

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Section: Mycology Bench Manual	Subject Title: Media / Reagents Brain Heart Infusion Agar	
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MEDIA / REAGENTS

2. BRAIN HEART INFUSION AGAR (BHIA)

Brain Heart Infusion Agar Powder	47 g. (Oxoid CM375)
Dist. H ₂ O	1000 ml.

Mix and boil to dissolve completely.
Distribute 10 ml. into 25-ml. UGB bottles.
Autoclave 15 minutes at 15 psi and 121⁰C.
Cool on a slant.
Store at room temperature.
Final pH approximately 7.4

Purpose: Isolation of opportunistic and dimorphic fungi from uncontaminated specimens.

Also to convert some dimorphic fungi from the mycelial phase to the yeast phase when incubated at 37⁰C with extra moisture added to the tube.

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3. CORNMEAL TWEEN 80 AGAR (OXOID)

Cornmeal	40 gm
Agar	20 gm
Tween 80 (polysorbate 80)	10 ml
Distilled water	1,000 ml

1. Mix cornmeal well with 500 ml of water; heat to 65⁰C for 1 hour.
2. Filter through gauze and then paper until clear. Restore to original volume.
3. Adjust to pH 6.6-6.8.
4. Add agar dissolved in 500 ml of sterile water.
5. Add Tween 80.
6. Autoclave for 15 minutes.
7. Dispense into sterile screw cap bottles (175 mls) to be melted and poured into petri dishes (15 ml/dish) as needed.

Purpose: For yeast morphology.

Reference

Davise H. Larone. Medically Important Fungi. A Guide to Identification. 1995, ASM Press.

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4. ESCULIN BASE MEDIUM (EBM) pH 7.1

Dist. H ₂ O	1000 ml.
Bacto Agar (Difco)	15 g.
Dextrose (BBL)	5 g.
Bacto-Peptone (Difco)	10 g.
Esculin (Difco/BDH)	0.5 g.
Difco Yeast Extract	1.0 g.

Mix thoroughly to dissolve.
Autoclave at 121°C/ 15 minutes

Cool to 45°-50°C, aseptically remove 5.0-ml. agar, then add:

2.5 ml. Gentamicin sulphate	= 25,000 µg/litre
2.5 ml. Chloramphenicol	= 10,000 µg/litre

Mix well and pour plates.
Store in fridge.

Gentamicin Sulphate Stock Solution (10,000 µg/ml)

Vial contains 2.0 ml. (40 mg/ml) = 80,000 µg

Transfer contents of vial and make up to a volume of 8 ml. using phosphate buffer pH 8.0 (= 10,000 µg/ml). Distribute 3 ml. amounts into bijou bottles. Store at -20°C.

Chloramphenicol Stock Solution (4,000 µg/ml)

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Purpose

Differential medium for isolation of *Cryptococcus neoformans* and also isolation medium for other fungi from contaminated specimens. Also provides presumptive identification of *C. neoformans*.

Principle

C. neoformans produces phenol oxidase enzyme that breaks down the substrate esculin, resulting in the production of a melanin-like pigment and the development of dark brown colonies. It takes about 48-72 hours for colonies to become brown. Other yeast colonies are cream to beige.

Rare strains of *C. neoformans* fail to produce pigmented colonies; also rarely yeasts other than *C. neoformans* produce dark colonies.

Quality Control

<u>Organisms</u>	<u>Incubation Temperature</u>	<u>Results</u>
<i>Cryptococcus neoformans</i>	28°C	Brown pigment
<i>C. laurentii</i> (or <i>T. glabrata</i>)	28°C	No brown pigment

References

S.C. Edberg et al. Esculin - Based Medium for Isolation and Identification of *Cryptococcus neoformans*. J. Clin. Micro. 12:332-335, 1980.

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5. INHIBITORY MOLD AGAR (IMA)

Tryptone	3.0 g.
Beef extract	2.0 g.
Yeast extract	5.0 g.
Dextrose	5.0 g.
Starch (soluble)	2.0 g.
Dextrin	1.0 g.
Chloramphenicol	0.125 g.
Salt A	10.0 ml.
Salt C	20.0 ml.
Agar	17.0 g.
Distilled water	970.0 ml.

Salt A:

NaH ₂ PO ₄	25.0 g.
Na ₂ HPO ₄	25.0 g.
NaH ₂ PO ₄ H ₂ O	28.71 g.
H ₂ O	250.0 ml.

Salt C:

MgSO ₄ - 7H ₂ O	10.0 g.
FeSO ₄ - 7H ₂ O	0.5 g.
NaCl	0.5 g.
MnSO ₄ - 7H ₂ O	2.0 g.
H ₂ O	250.0 ml.

Manufacturer: Que-lab Inc. Dehydrated medium.

Materials are dissolved in water that is brought to a boil to suspend the agar. After cooling, pH is adjusted to 6.7. Autoclave 15 psi/15 minutes. Chloramphenicol is first dissolved in 2 ml of alcohol (95%) and added to boiling media. Pour into sterile, plastic petri dishes (35 ml/plate). For isolation and subculture of fungi.

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Quality Control

<u>Fungus</u>	<u>Incubation Temperature</u>	<u>Result</u>
<i>Trichophyton mentagrophytes</i>	28°C	Good growth

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6. LACTOPHENOL ANILINE BLUE STAIN (LPAB)

Distilled water	20.0 ml.
Lactic acid	20.0 ml.
Phenol crystals	20.0 g.
Aniline blue	0.05 g.
Glycerol	40.0 ml.

Dissolve phenol in the lactic acid, glycerol, and water by gently heating. Then add aniline blue.

Purpose: Used for wet mount preparations of fungal cultures.

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7. MYCOSEL AGAR

Purpose

- a) to isolate pathogenic fungi (especially dermatophytes) from contaminated specimens (it inhibits bacteria and most saprophytic fungi).
- b) to determine Cycloheximide resistance of fresh isolates as a screening test for pathogenic fungi.

Dehydrated Mycosel Agar	36 g.
Distilled Water	1000 ml.

Mix thoroughly. Heat with frequent agitation until medium boils, not longer. Dispense 10 ml. amounts into 25-ml UGB bottles. Autoclave 15 min/ 118°C. Cool on a slant. Store at room temperature. Final pH 6.9 ± 0.2.

Formula per litre

Papaic Digest of Soybean Meal	10.0 g.
Dextrose	10.0 g.
Agar	15.5 g.
Cycloheximide	0.4 g.
Chloramphenicol	0.05 g.

Note: This medium must not be incubated at 35°C since the antibiotics at the higher temperature inhibit pathogenic fungi.

Quality Control

<u>Fungi</u>	<u>Incubation Temperature</u>	<u>Result</u>
<i>Trichophyton rubrum</i>	28°C	Good growth
<i>Aspergillus flavus</i>	28°C	No growth

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8. OXGALL AGAR

Purpose

For the rapid production of chlamyospore by *Candida albicans* within 24 to 48 hours.

Bacto oxgall powder	20 (10) g. (Difco 0128-02)
Bacto agar powder	18 (15) g. (Difco 0140-01)
Dist. H ₂ O	1000 ml.

Mix well. Final pH 7.3 ± 0.2.

Autoclave 121°C/15 minutes.

Cool to 50°C.

Pour plates.

Quality Control

<u>Fungi</u>	<u>Incubation Temperature</u>	<u>Results</u>
<i>Candida albicans</i>	28 ⁰ C	Chlamyospore production
<i>Candida tropicalis</i>	28 ⁰ C	No chlamyospore

Reference

J.B. Fischer, J. Kane "Production of Chlamyospores by *Candida Albicans* Cultivated on Dilute Oxgall Agar" Mycopath 35:223-229, 1968.

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9. POTATO DEXTROSE AGAR (PDA)

Purpose

Sporulation medium for fungi (can also be used in slide culture).

Slopes:

Potato dextrose agar (CM 139)	39 g.
Distilled Water	1000 ml.

Mix well, bring to a boil to dissolve. Cool to 50⁰C (check pH with ATC - pH ± 5.6). Dispense 10 ml amounts into pre-sterilised UGB bottles. Autoclave (with loose caps) at 118⁰C/10 minutes. Cool in a slanted position. Tighten the caps. Label the bottles "PDA". Store at 4⁰C.

Plates:

Potato dextrose agar	39.0 g.
Dist. H ₂ O	1000.0 ml.

Mix well, bring to a boil. Cool to 50⁰C (check pH with ATC - pH ± 5.6). Autoclave at 121⁰C/15 minutes. Cool. Pour plates (label "PDA"). Shrink wrap plates individually. Store at 4⁰C.

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10. SODIUM PYRUVATE AGAR (NPA) FOR NOCARDIA

Distilled Water	500 ml.
Sodium Pyruvate (Pyruvic acid. Sodium salt) SIGMA - P2256	2.5 g.
Yeast Extract	0.25 g.
Indicator (1.6 g bromocresol purple in 100 ml of 95% ethanol)	0.50 ml.

Mix well, pH to 6.8. Then add:

Agar (BACTO)	10 g.
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Mix thoroughly, heat to dissolve. Sterilize 121⁰C/15 min. Cool, pour plates. Store in refrigerator. (Final pH 6.8)

Purpose

Isolation medium for Nocardia from contaminated specimens. Growth of other bacteria is usually suppressed.

References

Saksun, J.M., Kane J. and Schaacter, R.K. 1978. Mycetoma caused by Nocardia madurae. CMAJ 119:911-914

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11. SABOURAUD AGAR MODIFIED (DIFCO)

Purpose

To provide a better and less inhibitory medium than the original formula for isolation and subculture of fungi.

Principle

The modified Sabouraud contains 2% rather than 4% dextrose and has a near neutral pH i.e. 7.0 as compared to 5.6. Other antibiotic containing media can now replace the very low pH in the original formula for suppressing bacterial contaminants.

Sabouraud Agar (Modified)	50 g.
Distilled Water	1000 ml.

Mix well pH 7.5

Heat to boiling to dissolve.

For slopes: Dispense 10 ml. amounts into UGB bottles. Autoclave 121°C/15 minutes. Slope. Store at RT. Final pH 7.0 at 25°C.

For plates: Autoclave flask 121°C/15 min. Cool. Pour plates. Store in plastic bags at 4°C.

Formula per litre

Bacto-neopeptone	10 g.
Bacto-Dextrose	20 g.
Bacto-Agar	20 g.

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12. SABOURAUD GENTAMICIN (50 mg/L) AGAR SLOPES

Purpose

To isolate fungi especially yeast from contaminated specimens.

Sabouraud Agar (Difco-modified-0747-01)	50 g.
Dist. H ₂ O	1000 ml.

Mix well, bring to a boil to dissolve. Cool to 50⁰C (check pH with ATC - pH ± 7.0). Add 1.25 ml Gentamicin (1 vial = 80 mgm/2 ml.). Mix well. Dispense 10-ml amounts into pre-sterilised UGB bottles. Autoclave 118⁰C/10 minutes. Cool in a slanted position. Tighten the caps. Label the bottles "SAB G". Store at 4⁰C.

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13. UREA AGAR SLOPES

Urea agar base* (Gibco M52600)	29 g.
Distilled Water	100 ml.

Sterilize by filtration.

Agar	15 g.
Distilled Water	900 ml.

Autoclave at 121⁰C for 15 minutes.

Cool to 50⁰C. Then add filtered urea agar base solution. Mix thoroughly and dispense 2.5 ml amounts aseptically into sterile disposable tubes (13 x 100) with white caps. Allow medium to cool in slanted position so that a deep butt is formed.

*Urea agar base is kept in the refrigerator.

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14. 10% POTASSIUM HYDROXIDE

Potassium hydroxide (KOH)	10g
Glycerol	20ml
Distilled Water	80 ml

Dissolve the potassium hydroxide in distilled water, then add glycerol. Mix well. Filter sterilize. Store in sterile amber bottle. Keep for 3 months.

Purpose

To digest or clear organic material e.g. tissue cells in a specimen in order to allow fungal structures to be more easily demonstrated.

Principle

Fungi are unaffected by KOH. Glycerol prolongs shelf-life by preventing crystallization and preserves the slides for a few days.