I. **PRINCIPLE**:

A. Specimens should be examined macroscopically and microscopically. The gross examination allows for selection of the proper portion of the specimen that will likely contain the fungus. Microscopic detection of a fungus in clinical specimens can alert the physician to the etiology of the disease and alert the laboratory staff to select the appropriate media and inoculation techniques that will enhance the recovery of the fungus.

B. Most specimens suspected of having fungi other than dermatophytes should be handled according to practices outlined for Biosafety Level 2.

II. **MACROSCOPIC EXAMINATION**:

Before inoculating a specimen to the appropriate isolation media, the specimen is examined macroscopically for caseous, purulent or bloody areas, and necrotic material. Specimens from cases of mycetoma are examined with the dissecting microscope for the presence of granules before proceeding. Punch biopsies that must be split for other lab procedures are examined closely to ensure that they are divided vertically and not horizontally so that each layer of tissue is represented in each specimen.

III. **MICROSCOPIC EXAMINATION**:

A. Specimens of any clinical nature can be examined microscopically for fungi upon the physician's request or at the initiative of the laboratorian. All specimens of sufficient quantity submitted for fungal culture should be examined microscopically for fungi. When there is not a sufficient quantity of a specimen for both a culture and direct exam, the culture takes priority over the smear because it is more sensitive than microscopic examination. Observing a fungus in a clinical specimen (Table 2) is, however, often valuable in establishing its significance and in providing early information that may be crucial for determining appropriate therapy for the patient.

1. Smears should be prepared from

    a. specimens submitted for an express diagnosis of fungal disease: tissue, aspirates, respiratory specimens (urine, if diagnosis is histoplasmosis, blastomycosis, or coccidioidomycosis).
    b. specimens from transplant patients: tissues, aspirates, respiratory specimens.
    c. specimens from patients with a diagnosis of neutropenia, acute leukemia, HIV infection: tissue, aspirates, respiratory specimens.
    d. specimens from neonates: any specimen other than feces.

2. If a specimen is submitted for fungal culture and the patient history does not reflect one of the above clinical conditions, and if policy dictates that the physician must request a direct examination, the laboratorian should contact the appropriate personnel to make such a request.
3. One specimen for which microscopic examination is not recommended is feces. In humans, the only detectable fungus of probable significance is *Candida* (3). However, in dogs with a history of chronic diarrhea, a rectal biopsy smear may yield evidence of *Histoplasma* and is, therefore, a valuable tool for diagnosis.

III. **MICROSCOPIC EXAMINATION**: (cont'd)

B. Preparation of Specimen for Microscopic Examination:

1. Tissue should be thoroughly minced with a scalpel (3). The calcofluor and KOH penetrate slowly or not at all into the interior of inadequately minced pieces.
2. Fluids (CSF, urine, pleural, peritoneal, joint) should be concentrated by centrifugation at 1500-2000 g (3).
3. Nail clippings can be progressively shaved with a scalpel and pulverized with mortar and pestle.

C. Methods of Microscopic Examination:

1. Potassium Hydroxide Procedure

a. Principle: KOH may be used to examine hair, nails, skin scrapings, fluids, exudates, or biopsies. The fungal structures such as hyphae, large yeast (*Blastomyces*), spherules, and sporangia may be distinguished. Examine slides with reduced light (narrow the iris diaphragm) and examine negative smears on several consecutive days. In unstained preparations (KOH without ink or specimen with no reagent), the fungal structures may be enhanced by using a phase-contrast microscope. Specimens placed in a drop of 15% KOH will dissolve at a greater rate than fungi because fungi have chitinous cell walls. The clearing effect throughout the clinical specimen can be accelerated by gently heating the KOH preparation. Visualization of fungi can be further enhanced by the addition of Parker Superquink permanent black ink to the preparation.

b. Specimen: The specimen may consist of all types of clinical material. Fluids such as CSF generally do not need to be treated with KOH and a Cryptococcus latex test will be suggested instead.

c. Preparation of reagents:

(1) 15% Potassium Hydroxide Solution

(a)	potassium hydroxide	15 g
(b)	glycerol	20 ml
(c)	distilled water	80 ml

(2) Potassium hydroxide plus Parker's Ink solution

(a)	solution in 3.1	8 ml
(b)	Parker Superquink permanent black ink	2 ml

(3) Store solutions at 25°C.

d. Quality control:

(1) Check the reagents prior to use. If cloudy, prepare new solution.
(2) Using a portion of a sputum specimen, insure that the clinical material dissolves.

e. Procedure:

(1) Place the material to be examined onto a clean glass microscope slide.
(2) Add a drop of 15% KOH to the material and mix.
(3) Pace a cover glass over the preparation.
(4) Allow the KOH prep to sit at room temperature until the material has been cleared. The slide may be warmed to speed the clearing process. Slides that are initially negative for fungi may be re-examined the following day.
(5) Observe the prep by brightfield or phase-contrast microscopy. The illumination on a brightfield microscope should be carefully adjusted using the Kholer method. Hyaline fungi will be difficult to see if the illumination is improperly adjusted. Refer to the table in the EXAMINATION OF SPECIMENS for interpretation of positive results.

f. Results: As the clinical specimen clears as it dissolves, fungi will be destroyed in KOH preparations in time. This technique does not result in permanent preparations. Refer to COORDINATION AND RESULTING section for significance of positive results.

g. Additional reading:

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

2. Potassium Hydroxide - Calcofluor White Solution Mixture

a. Principle - Calcoflour white stain may be used for direct examination of most specimens using fluorescent microscopy. The cell walls of the fungi bind the stain and fluoresce blue-white or apple-green depending on the filter combination used (1). The use of calcofluor white (CFW), a fluorescent brightener used in the textile industry, with the addition of potassium hydroxide (KOH) will enhance the visualization of fungal elements in specimens for microscopic examination. The CFW non-specifically binds to the chitin and cellulose in the fungal cell wall and fluoresces a bright green to blue. A substantial amount of non-specific fluorescence from human cellular materials and natural and synthetic fibers should be expected. The CFW highlights suspicious structures but the interpretation of the structures relies on traditional fungal morphologic features. KOH-CFW preparations may be preserved for several days at 4°C in a humid chamber.
b. Specimen: The specimen may consist of all types of clinical material.
c. Preparation of reagents:

(1) 15% Potassium Hydroxide Solution

(a) potassium hydroxide 15 g
(b) glycerol 20 ml
(c) distilled water 80 ml
(d) Store at 25°C and discard if a precipitate forms.

III. **MICROSCOPIC EXAMINATION**: (cont'd)

(2) 0.1% Calcofluor White (W/V) Solution

(a) Use commercially available solution cellufluor 17352 (Polysciences, Washington, PA), fluorescent brightener 28.F6259 (Sigma, St. Louis, MO), 1 gm
(b) distilled water, 100 ml
(c) Gently heat if precipitate develops. Filter if precipitate persists. Store at 25°C in the dark.

(3) Commercially prepared kits are available and suited for the procedure.

d. Quality control:

(1) Check the reagents prior to use, weekly, and with each new batch of calcofluor prepared.
(2) Using an aqueous suspension of actively growing *Candida albicans*, the yeast cell walls will be bright green to blue-white depending upon ultraviolet filters used.
(3) Negative control consists of KOH and calcofluor combined.
(4) Record on proper QC sheet.

e. Microscope filter system: An epifluorescent microscope equipped with a mercury vapor lamp and either an ultraviolet (UV) or blue-violet (BV) excitation filters to achieve radiation on the slide below 412 nm should be used since the maximum absorbance of CFW is 347 nm. Several brad band or barrier filters are available to achieve the radiation required (300-412 nm) from the microscope manufacturers. Depending on which barrier filter is used, a blue-white or a bright green color will be observed staining the fungal cell walls. A microscope with selective filters that will prevent radiation below 490 nm should not be used for CFW; neither should a microscope with a quartz halogen bulb since the energy output is so low.

f. Procedure:

(1) Place the material to be examined onto a clean glass microscope slide.
(2) Add a drop of 15% KOH and a drop of the CFW solution, or mix in equal volumes before processing.
(3) Mix and place a cover glass over the material.
(4) If necessary, allow the KOH preparation to sit at room temperature (25°C) for a few minutes until the material has been cleared. The slide may be warmed to speed the clearing process.
(5) Observe the prep by UV/BV microscopy. CFW may not stain strongly dematiaceous fungi that cause chromoblastomycosis or dark-grain mycetoma. If such is suspected, the preparation should also be examined using brightfield microscopy. *Torulopsis glabrata* may fluoresce only very faintly. Elastin and collagen will also fluoresce, but with a yellow-breen fluorescens. These will be distinguished by their morphology. Use the table in the EXAMINATION OF SPECIMENS for interpreation of positives.

g. Results: Fungi stain bright green or blue-white depending upon the filters used. Refer to COORDINATION AND RESULTING section for significance of results.

III. **MICROSCOPIC EXAMINATION**: (cont'd)

h. Additional reading:

(1) Hageage, GJ, Harrington, BJ: Use of calcofluor white in clinical mycology. Lab. Med. 15:109-112, 1984.
(2) Harrington, BJ, Hageage, GJ: Calcofluor white: tips for improving its used. Clin. Microbiol. Newsletter 13(1):3-5, 1991.

3. India Ink - India ink can be added to specimens such as spinal fluids or exudates to provide a dark background that will highlight hyaline yeast cells and capsular material (halo effect). Hence, it should be used to examine specimens suspected of containing *Cryptococcus neoformans*. White blood cells may be distinguished from *Cryptococcus neoformans* because of the irregular edge of the halo and the pale cell wash (3). The India ink preparation is not routinely offered by the laboratory. If a request is received for it, the laboratory should call the physician and offer a Cryptococcus Antigen Test instead. The procedure will be performed only in particular instances with the approval of the director or supervisor.

4. Buffy Coat for *Histoplasma capsulatum* - Hematological smears may be used for examining specimens for *Histoplasma* which usually occurs as an intracellular yeast. Fluids, exudates, or buffy coats should be spread evenly over a slide and impression smears may be prepared from tissues. Stain according to directions and examine using 40x and 100x magnification. Diff-Quick, Wright stain, or Giemsa stain may be used. For procedure and quality control records, refer to the hematology procedure manual. The laboratory will accept stained slides after they have been processed by Hematopathology for examination of the presence of *Histoplasma capsulatum*. A consultation form should be attached to the request and all slides should be reviewed by the director.

5. Gram Stain - Gram stain is usually a poor stain to use when examining a specimen for a fungus. An experienced technologist may be able to presumptively diagnose evidence of a fungus and make the decision to do other stains; however, an inexperienced technologist may completely overlook the evidence. Gram stain may be used when examining smears of *Candida*, *Malassezia*, and *Sporothrix* but should not be relied upon to demonstrate the yeast of the other dimorphic fungi. A gram stain will demonstrate the filaments of *Nocardia* and *Actinomyces* which may produce clinical signs resembling mycotic infections.

6. Modified Kinyoun Acid Fast Stain for *Nocardia* -

a. Make a thin smear of the specimen to be stained; fix in methanol. A positive control smear (*Nocardia asteroides*) and a negative control smear (*Streptomyces sp.*) must be included.
b. Kinyoun carbolfushcin; 5 minutes, no heat.
c. Rinse with water.
d. 50% ethanol rinse; flood and pour off until excess carbolfuchsin is removed.
e. Rinse with water.
f. Decolorize with 0.5% (aqueous) H_2SO_4; 3 minutes.
g. Rinse with water
h. 1% (aqueous) methylene blue; 1 minute.
i. Rinse with water.

III. **MICROSCOPIC EXAMINATION**: (cont'd)

j. Blot or air dry.
k. Examine smear under oil immersion.
l. Record QC results on proper worksheet.

IV. **REFERENCES**:

1. Baron, EJ, Finegold, SM: Baily & Scott's Diagnostic Microbiology, 8th Edition, St. Louis, C.V. Mosby Company, 1990.

2. Koneman, EW, Roberts, GD: Practical Laboratory Mycology, Baltimore, The Williams and Wilkins Co., 1985.

3. Larone, DH: Medically Important Fungi, A Guide to Identification, 2nd Edition, New York, Elsevier Science Publishing Co., 1987.

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

5. Rippon, JW: Medical Mycology: The Pathogenic Fungi and the Pathogenic Actinomycetes, Philadelphia, W. B. Saunders Co., 1988.

Table 1. Morphology and Possible Fungi

Hair	nodules present	black, hard		*Piedraia*
		soft, white		*Trichosporon*
	nodules absent	arthroconidia present	ectothrix	*Microsporum* *Trichophyton*
			endothrix	*Trichophyton*
		arthroconidia absent		*Trichophyton*
Nail	hyphae	dematiaceous		*Scytalidium*
		non-dematiaceous		*Aspergillus* *Epidermophyton* *Microsporum* *Scopulariopsis* *Trichophyton*
	hyphae, pseudohyphae, yeast cells			*Candida*
	short sinous hyphae and yeast clusters			*Malassezia*
Stratum corneum	hyphae	dematiaceous		*Phaeoannellomyces*
		non-dematiaceous		*Candida* *Epidermophyton* *Microsporum* *Scytalidium* *Trichophyton*
	hyphae, pseudohyphae, yeast cells	dematiaceous		*Phaeoannellomyces*
		non-dematiaceious		*Candida*
	Yeast Cells	globose	multiple budding	*Paracoccidioides*
			single blastoconidium	broad attachment	*Blastomyces*
				narrow attachment	*Cryptococcus* *Histoplasma*
		ovoid	4-7 um		*Candida*
			2-3 um		*Candida* *Histoplasma* *Malassezia* *Sporothrix* *Torulopsis*

Stratum corneum (continued)	Hyphae	6-10 um, broad, irregular			*Candida*
		3-4 um	dematiaceous		*Bipolaris* *Exophiala* *Phialophora* *Xylohypha*
		3-4 um	non-dematiaceious		*Aspergillus* *Candida*
		0.5-1.0 um filamentous			Actinomycetes
	Pseudohyphae, yeast cell				*Candida*
	Sporangia, 100-200 um				*Rhinosporidium*
	Sherules, 30-60 um				*Coccidioides*
	Sclerotic bodies				*Cladosporium* *Fonsecaea* *Phialophora*
	Adioconidia				*Chrysosporium*
	Granules	globose cells, dematiacious			*Exophiala*
		coccoid forms			bacteria
		filamentous	0.4 - 1 um		Actinomycetes
			3-4 swollen cells		*Acremonium* *Madurella* *Scedosporium*
	Chain of globose cells				*Loboa*
Fluids Exudates Biopsies	Yeast cells	globose	multiple budding		*Paracoccidioides*
			single blastoconidium	broad attachment	*Blastomyces*
				narrow attachment	*Cryptococcus* *Histoplasma*
		ovoid	4-7 um		*Candida*
			2-3 um		*Candida* *Histoplasma* *Sporothrix* *Torolopsis*
	Hyphae	6-10 um, broad, irregular			*Rhizopus*
		3-4 um		dematiaceous	*Bipolaris* *Exophiala* *Phialophora* *Xylohypha*
				non-dematiaceous	*Aspergillus* *Candida*

Fluids Exudates Biopsies (continued)	Pseudohyphae, yeast cell			*Candida*
	Sporangia, 100-200 um			*Rhinosporidium*
	Spherules, 30-60 um			*Coccidioides*
	Sclerotic bodies			*Cladosporium* *Fonsecaea* *Phialophora*
	Adioconidia			*Chrysosporium*
	Granules	globose cells, dematiaceous		*Exophiala*
		Ccoccoid forms		Bacteria
		filamentous	0.5 - 1 um	Acetinomycetes
			3-4 swollen cells	*AcremoniumMa durella* *Scedosporium*
	Chain of globose cells			*Loboa*