Procedure for Preserving Yeast and Mold Isolates

1. Purpose

This document outlines the procedure for preserving yeast and mold isolates for storage. Preserving yeast and mold isolates ensures long-term viability.

2. Scope

This document will describe procedures to follow when preserving yeast and mold isolates. The preservation method to be used will depend on an individual isolate's morphology and sporulation. Non-sporulating molds can be frozen, but the long-term viability of the isolate may be greatly decreased.

3. Responsibilities

Position	Responsibility
Laboratory Technicians	• Subculture the isolates • Prepare and mount slide cultures • Check for sporulation and purity • Perform the procedure
Laboratory Supervisor	• Ensures laboratory personnel are properly trained in freezing and transporting yeast and mold isolates

4. Definitions

Term	Definition
SAB Media	Sabouraud's dextrose agar
YPSS Media	Yeast starch agar
SCG Media	Sabouraud's dextrose agar with chloramphenicol and gentamicin
PD	Potato dextrose agar
BSC	Biological Safety Cabinet

5. Equipment

5.1. Microscope

5.2. Biological Safety Cabinet

5.3. Incubator

5.4. -80°C Freezer

5.5. Refrigerator

6. Reagents/ Media

6.1. **80 % Glycerol-Sterile Solution:** (*ex*: MP Biomedical Catalog # 3055-044 or equivalent). Always use glycerol labeled "for cell culture use" or "molecular biology reagent". Chemical-grade glycerol is not acceptable.

6.2. **Sterile WFI Quality -Cell Culture Grade Water**

6.3. **Sabouraud Dextrose Agar (Emmons):** (*ex*: Fisher Scientific Product# L4321849 and L21827 or equivalent).

6.4. **Sabouraud Dextrose Agar and Chloramphenicol/Gentamicin:** Slants (*ex*: Fisher Scientific Product #R0876 or equivalent).

6.5. **Oatmeal/Pablum agar:** (always use a mixed cereal powder, not straight oatmeal or rice powder). This can be found in the baby food aisle of the grocery store.

6.6. **Potato Dextrose Agar:** (*ex*: Becton Dickinson catalogue #213200 or equivalent).

6.7. **Tween-20:** (*ex*: Fisher Scientific Product# BP337-100 or equivalent).

6.8. **Lactophenol Cotton Blue:** (*ex*: Fisher Scientific Product# M1137410100 or equivalent).

7. Reagent Preparation

7.1. **500 ml solution of 20% Glycerol in Sterile Water** – <u>Prepare ahead of time to be ready for procedures below.</u>

7.1.1. Add 375 ml of sterile WFI quality cell culture grade water to a 1000 ml flask.

7.1.2. Add 125 ml of 80% glycerol to the 1000 ml flask containing water.

7.1.3. Stir the solution until the ingredients are mixed thoroughly.

7.1.4. Filter the solution into a Nalgene sterile filtration unit-0.22 pore size.

7.1.5. Label the bottle as 20% Glycerol and include the date and preparers name/initials and store at 4°C.

7.2. <u>Oatmeal/Pablum agar:</u>

7.2.1. Add 25 g of a mixed cereal powder (always use a mixed cereal powder, not straight oatmeal or rice powder. This can be found in the baby food aisle of the grocery store) to 200 mL of deionized water.

7.2.2. Add 5 g of agar.

7.2.3. Adjust the final volume to 250 mL.

7.2.4. Autoclave for 20 minutes and dispense to petri dishes. The agar should be thin, approximately 3-5 mm. Should make 10-12 plates.

8. Supplies, Other Materials

8.1. 10 µm Sterile inoculating loops

8.2. Cryogenic freezer boxes

8.3. 15 ml blue top conical Falcon tubes

8.4. 10 ml serological pipettes

8.5. Microscope slides

8.6. Any size cover slips

8.7. 1000 ml glass flask

8.8. 4-Chamber petri dish

8.9. Lactophenol cotton blue or other stain

8.10. Sterile scalpels

8.11. Sterile Cotton Swabs

8.12. Box of Sterile Needles (18G1)

8.13. 0.22-micron filter

8.14. Cryogenic vial 2 ml microfuge tubes

9. Safety Precautions

NOTE: All institutional safety procedures must be followed in the performance of this standard operating procedure.

9.1. Standard personal protective equipment should follow institutional guidelines but should consist of at least gowns, gloves, and safety glasses.

9.2. Samples should be handled as if infectious using safe laboratory procedures such as those outlined in Biosafety in Microbiological and Biomedical Laboratories 5th Edition and in the CLSI Document M29-A.

9.3. Thoroughly clean and disinfect all work surfaces with a freshly prepared solution of Lysol Brand I. C. Quaternary Disinfectant Cleaner diluted 1:128 with distilled water.

9.4. Wash hands thoroughly after handling samples and test reagent.

9.5. Do not pool reagents from different lots or from different bottles of the same lot.

9.6. Dispose of unused reagents and waste in accordance with federal, state, local and institutional regulations.

10. Procedure

10.1. Freezing Yeast Isolates

Step	Action
1	Remove a fresh SCG agar plate or slant from the 4°C refrigerator and place on the benchtop. Always allow the plate or slant to warm to room temperature.
2	Streak the SCG plate or slant by taking a light inoculum from the yeast culture to be stored and inoculate a fresh plate.
3	Incubate the inoculated plate for 48 hours at 30°C. Culture must be in mid- to late-log phase growth. Adjust incubation time as needed until 2-3mm colonies are seen.
4	Check the isolate microscopically to verify that it is free of bacterial contamination. You should only see yeast cells under 40x.
5	The **following steps** should be **performed in a BSC** to ensure sterility.
6	Prepare screw capped cryogenic vials labelled with the date and isolate identification name/number. Use a printed label whenever possible. Printed labels should be sufficiently robust to tolerate temperature of -80°C without disintegrating. Handwriting should be legible and ink robust to tolerate -80°C.
7	Add 1.5 ml of sterile 20% glycerol to each vial.
8	Make a heavy suspension of the yeast growth using a sterile inoculating loop or cotton swab by emulsifying the yeast growth into each vial. Secure the lid and vortex for 5 sec.
9	Store vials at -70°C or as appropriate for your laboratory.

10.2 Alternate Method: Freezing Yeast Isolates

Step	Action
1	Follow Steps 1 through 4 in freezing method 10.1.
2	The following steps should be **performed in a BSC** to ensure sterility.
3	Prepare screw capped cryogenic vials labelled with the date and isolate identification name/number. Use a printed label whenever possible. Labels should be sufficiently robust to tolerate temperature of -80°C without disintegrating. Handwriting should be legible and ink robust to tolerate -80°C.
4	Using aseptic technique, pipette 9 mL of sterile 20% Glycerol solution to a sterile15 mL graduated conical tube.
5	Make a heavy suspension of the yeast growth using a sterile inoculating loop or cotton swab by emulsifying the yeast growth (approximately 5 colonies) into the 15 mL graduated test tube. Secure the lid and vortex for 5 sec.
6	Using a sterile transfer pipette, transfer approximately 1.5 ml of the yeast suspension to each cryogenic vial.
7	Store vials at -70°C or as appropriate for your laboratory.

Note: The method described below in section 11.1 can also be used for yeast isolates.

11.1 Freezing Mold Isolates with heavy sporulation

Step	Action
1	Remove a fresh slant of solid medium (SA, PD etc.) from the 4°C refrigerator, allow to warm to room temperature and place in the BSC. **Note**: Different groups of organisms sporulate better on different types of media (See **Table A** below)
2	Make sure there is enough volume of 20% Glycerol for the number of isolates to be frozen. If not, go to step 7.1.
3	Place the mold to be preserved in the BSC.

4	Inoculate a fresh slant and place in the incubator at 25°C. **Note:** There are a few molds that require a different temperature for spore production.
5	Incubate slant for at least 7 days or until luxurious conidial production is observed microscopically. Slow growing organisms may require longer incubation. Fast growing organisms will require less than 7 days.
6	Perform a tease preparation of the slant to confirm spore production. Verify that the culture is not contaminated with bacteria.
7	**Note**: View the culture microscopically and look for the production of spores/conidia. If sporulation does not occur, refer to the procedure for selective sporulation medium (Section 11.3).
8	a. Prepare screw capped cryogenic vials labelled with the date and isolate identification name/number. Use a printed label whenever possible. Labels should be sufficiently robust to tolerate temperature of -80°C without disintegrating. Handwriting should be legible and ink robust to tolerate -80°C. b. Using a 10 ml sterile pipet add 8 ml or less of 20% glycerol to the slant containing the mold to be stored. Isolates that do not show sufficient growth will require fewer tubes and smaller volume. c. Using a 1ml sterile transport pipet or a loop, make a heavy suspension by emulsifying the mold spores in the 20% glycerol by lightly scraping the mold growth with the pipet and by repeatedly drawing in and expelling the glycerol. d. Use the transport pipet to add ~1.5 ml of the suspension into each of the cryovials and secure the lids. e. Store vials at -70°C or as appropriate for your laboratory.

11.2 Table A. Recipes for these media, if necessary, can be found in fungal textbooks.

Organism	Sporulation medium	Alternate sporulation medium
Dermatophytes	**Pablum**	**Leonian's**
Aspergillus/Penicillium spp.	**Czapek**	**Malt**
Mucormycetes	**PDA**	**Tap water**
Black molds	**Pablum**	**V-8**
Fusarium sp.	**PDA**	**Potato-carrot**
Coccidioides sp.	**YPSS**	**Pablum**

NOTE: Slants should be incubated at 25°C and checked periodically (twice weekly) for up to 2 weeks. Some organisms will require longer incubation or incubation at 37°C depending on the species.

11.3 Freezing Mold Isolates that do not sporulate well (must perform in a BSC)

Step	Action
1	Remove a fresh plate of solid medium (consult **Table A**) from the 4°C refrigerator, allow it to warm to room temperature and place in BSC.
2	Make sure there is enough volume of 20% Glycerol for the number of isolates to be frozen. If not, go to step 7.1.
3	Place the mold to be preserved in the BSC.
4	Inoculate the fresh plate and place in the incubator at 25°C. **Note:** There are a few molds that require a different temperature for spore production.
5	Hold the plate for at least 7 days. Slow growing organisms may require longer incubation.
6	**Note:** For a very slow growing isolate it may be necessary to inoculate multiple plates.
7	Perform a tease preparation of the colony to look for spore production Verify that the isolate is not contaminated with bacteria.

8	**Note:** Spore production may be scant; By harvesting large sections of the fungal growth which may contain some of the few rare spores, plates may be used with scant spore production.
9	a. Prepare sterile screw-capped cryogenic vials labelled with the specific specimen number and date. A printed label should be used whenever possible. Labels should be sufficiently robust to tolerate temperature of -80°C without disintegrating. Handwriting should be legible and robust to tolerate -80°C. b. Using a sterile pipet, add 1.5 ml of 20% glycerol to each vial. c. Using a sterile scalpel and cut small (3-4 mm) square blocks of fungal mat from the outer (actively growing) edges. Avoid taking the agar medium. d. Add 5-6 blocks of fungal mat to each vial and make sure they are immersed in the glycerol. **e.** Store vials at -70°C as appropriate for your laboratory.

11.4 Preserving Molds in Water Stock (must be performed in the BSC)

Step	Action
1	Grow the organisms in appropriate sporulation medium. (consult **Table A**).
2	Check for sporulation and purity following the above procedures (**11.3**)
3	a. Prepare sterile screw-capped cryogenic vials and label them as stated above with the specific specimen number and date (11.3, step 9a.) b. Fill the slant containing the mold to be stored with 8 ml of sterile distilled water (water should be previously sterilized by filtration or autoclaving) and one drop of an emulsifying agent such as Tween-20. c. Using a 1ml sterile transport pipet or a loop, make a heavy suspension by emulsifying the mold spores in the sterile water. This is accomplished by lightly scraping the mold growth with the pipet and by repeatedly drawing in and expelling the sterile distilled water. d. Add ~1.5 ml of suspension into each cryovial and secure lids. e. Place the vials in the appropriate labeled box and store at room temperature.

11.5 Preserving Mucormycetes and dematiaceous organisms in water (must be performed in the BSC)

Step	Action
1	a. Use a sterile 25ml pipet to add 25ml of sterile tap water to a petri dish (tap water is sterilized by filtration through a 0.22 filter) b. Use a pasteur pipet to add 3 or 4 drops of sterile 10% yeast extract to the dish c. Take a sterile needle (18G) to pick a few mold colonies and add them to the petri dish. Mix the colonies with the other ingredients by rotating the needle gently in a circular motion. d. Incubate the petri dish in the 37°C incubator for 3 days to 2 weeks (depending on the species and the growth of the organism).
2	After growth, check for sporulation and purity by removing a small colony and making a tease prep.
3	a. Prepare sterile screw-capped cryogenic vials and label them as stated above with the specific specimen number and date. b. Use a sterile needle or pipette to pick 2-3 colonies from the petri dish and add them to the cryovial. c. Using a sterile 1 ml transfer pipet to add sterile distilled water to a total volume of 1 ml and mix up and down with the transfer pipet. d. Place the vials in the appropriate labeled box.

DISCLAIMER:

The Mycotic Diseases Branch laboratory developed this document as an example test procedure for preserving yeast and mold isolates for storage. It is the responsibility of the testing laboratory to ensure content and format are modified as necessary to meet applicable regulatory requirements, quality management system standards, and chemical, radiological and biological safety requirements. This is not a controlled document and the described test methods are subject to change without notice. It is the responsibility of the testing laboratory to ensure the information within this document remains applicable. Contact the test developer at gyi2@cdc.gov to find out whether any changes have been made.
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