Title: Preparing Glycerol Stocks			
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Preparing Glycerol Stocks

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this document is to set forth **standard** guidelines for preparing glycerol stocks.
Freezing bacterial isolates down requires good sterile technique to ensure you are freezing only the intended organism; if you have never been taught aseptic technique, please stop what you are doing and see a technician.

1.2 Scope

This SOP applies to the Food Safety Lab and MQIP Lab

1.3 Definitions

1.4 Safety

L. monocytogenes, E. coli, and *S. enterica* are BSL-2 pathogens. Appropriate safety precautions including the utilization of PPE (lab coats, gloves, and safety glasses) must be observed when working with these organisms. Any waste generated must be treated as BSL-2 waste.

When working with a flame, ensure the area around the burner (360°) is clear. There have been instances where the glass in the cabinet doors above the burner has cracked due to the heat rising from the flame. Turn off the flame if you leave the bench.

SECTION 2 MATERIALS

- Plated pure cultures containing well-formed isolated single colonies.
- BHI liquid media, 5ml tubes lab stock, room 350 supply cabinets.
- Sterile wood inoculating sticks, lab stock room 350 supply cabinets.
- Bunsen burner
- 70% ETOH
- 100% Glycerol, lab stock, room 350 supply cabinets.
- Incubator set at appropriate temperature for the bacteria
- Pipette
- Sterile Pipette tips, lab stock, room 350 supply cabinet
- 1.5 mL screw cap tubes containing rubber washers and cap inserts
- Glass tube rack
- Microtube rack

SECTION 3 PROCEDURES

3.1 Preparation of overnight suspension culture

(1) Disinfect your bench and pipetman
(2) Label your BHI tubes with the corresponding FSL numbers.
(3) Turn on Bunsen burner
(4) Open the tube containing the inoculation stick, and flame the top
(5) Select a stick, and flame the tip to be used to pick the colony, allow the stick to cool for a few seconds
(6) Remove the cover of the petri plate and using your sterilized stick, pick an isolated colony
(7) Remove the cap from the labeled BHI tube, and flame the opening.
(8) Aseptically transfer the colony from the stick to the broth, take care to touch only the broth with the stick.
(9) Re-cap the tube, and repeat for remainder of the isolates to be prepared.
(10) Incubate your suspensions at the temperature appropriate for your culture (i.e. 35°C for *Listeria)* overnight.
    a. "Overnight" times are dependent on the organism. For most robust organisms 15-18 hours is adequate. Do not overgrow. The goal is to freeze down bacteria during late log or early stationary phase. Always inoculate a negative control when growing overnight suspensions.

3.2. Preparation of glycerol stocks:

(1) Disinfect your bench, pipette, and tube rack with 70% ETOH before you begin
(2) Label the sides of your microtubes with the FSL number, genus, species, date and your initials, and the cap inserts with the FSL number so that they can be easily found in the freezer box.
(3) Loosen cap & microwave a small bottle of sterile glycerol for 10 seconds to make it easier to pipet. Gently agitate glycerol to mix. Do not overheat the glycerol! While glycerol is not flammable, it will burn.
(4) Working aseptically, aliquot 150ul of warm, sterile glycerol into each cryovial at an angle and replace cap. Do not remove cryovial caps and place on bench!
(5)) After allowing glycerol to cool down for a few minutes, vortex your overnight culture briefly and aseptically add 850ul of overnight culture to its appropriate cryovial.
(6) Invert each cryovial until glycerol and overnight culture appear mixed
(7) Immediately place cryovials into your –80 freezer box.
(8) Log isolate locations into Food Microbe Tracker for the tower and box.

Note: It is recommended that you freeze down no more than 40 isolates at one time. If you do freeze down a large batch of isolates, place tubes on ice after mixing and invert again several times before placing in freezer. Do not let isolates sit in glycerol at room temperature.

3.3 Confirmation of cultures

(1) This is a general SOP typically used with robust organisms (Listeria, Salmonella, E. coli and Pseudomonas). If you are working with a new or unknown organism, you should confirm the viability of your frozen stock before throwing out your plates.

a. Select random isolates from the glycerol stocks two or three days after they were frozen.

b. Sub-culture on medium specified for your organism.

c. Incubate your cultures at the appropriate temperature for your culture (i.e. 35°C for *Listeria)* overnight.

d. Confirm your organism grew as expected, is a pure culture, and has the correct phenotype

SECTION 4 TROUBLESHOOTING

(1) Overnight suspensions did not grow
   a. Confirm you are using the correct growth medium for your organism
   b. When inoculating the suspension, be sure to allow the stick to cool. If you pick a colony before the stick has cooled down, you have likely injured/killed your organism.
   c. Confirm the incubation conditions for your organism.
   d. Confirm the incubator is operating correctly.

(2) Suspensions do not appear homogeneous after vortexing
   a. The suspension may be contaminated. Discard this, and prepare a new suspension per section 3.2, confirming that your initial plate culture is pure.

(3) Confirmation plates are not pure
   a. A contamination occurred during the preparation of the glycerol stock or overnight suspension.
      i. Confirm your original plate culture is pure
      ii. Discard this stock and repeat the process from 3.1.

(4) Confirmation plates have scant/no growth
   a. Inadequate mixing of suspension can cause the glycerol stock to be of low concentration.
      i. Discard this stock and repeat the process beginning from section 3.1. Insure your suspension is well mixed before adding it to the glycerol.
   b. Inadequate mixing of the glycerol stock before freezing can affect how well the subculture grows.
      i. Since the freeze/thaw process effects the quality of the stock, a new stock should be prepared.

(5) In cases where isolates are found to be non-viable or misidentified after assigning an FSL number, discard the glycerol stock, inactivate the entry in Food Microbe Tracker, make a notation in your notebook, and retire the number. DO NOT reassign this number for future isolates; it's OK to have gaps in numbering.

SECTION 5 REFERENCES

SECTION 6 METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1	06/27/2016		Original SOP
Version 2	04/16/2020	Ser15	Formatted SOP into new template. Split glycerol stock procedure from FSL number assignment, added trouble shooting section.