Standard Operation Procedure (S.O.P.) for the Preparation of Bacterial Glycerol Stock

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This S.O.P. will direct you in preparing bacterial glycerol stocks, which allow long-term storage of bacteria strains containing recombinant plasmids, thus, offering a method for storing expression vectors that could be easily amplified and purified. Adding glycerol stabilizes the frozen bacteria, preventing damage to the cell membranes and keeping the cells alive.

- *The glycerol stocks can last many years (<10 years).*
- *The optimal glycerol concentration is unknown. 15–20% is mainly used.*
- *Try not to freeze/thaw your glycerol stock too many times. Placing the glycerol stock on dry ice while streaking onto LB agar will prevent it from thawing entirely and improve the shelf life.*

Important:

- ***Snap top tubes are not recommended for storage at -80 °C as they can open unexpectedly. Instead, use a screw top tube or cryovials.***
- ***All volumes are given on a per-culture tube basis.***

Materials & Equipment:

- 70% Ethanol
- Micropipettes and Tips for 200 and 1000 μL
- Micropipettes and Tips or Serological Pipettes for 5 mL
- Reverse Osmosis Water (RO Water)
- Waste Container with Concentrated Bleach (2% Sodium Hypochlorite)
- 7–14 mL Round-Bottom Culture Tubes
- 1.0–1.8 mL Cryovials
- LB Media Containing the Appropriate Antibiotics and Chemicals (stored at 4 °C)
- 50% Glycerol Solution
- Incubator with Orbital Shaker at 37 °C and 225 r.p.m.
- Spectrophotometer Cuvette
- Spectrophotometer with 600 nm Filter
- Vortex

Procedure:

<u>*Day Before*</u>:

a) The working area and materials must be sanitized using 70% ethanol before placing anything on the bench.

b) Prepare the waste container with bleach for decontamination. ***Any material in contact with intact bacteria must be decontaminated in this container for at least 30 minutes***.

c) Determine how many culture tubes will be purified and label the tubes properly. For each culture tube (14 mL tube), add the appropriate volume of LB media with the appropriate antibiotic (***see table below***). Inoculate the media with an isolated bacterial colony from the agar plate using an inoculation loop. Grab the inoculation loop, touch it the less possible, and submerge it into the media. Be careful not to touch the tube walls. Agitate the media with the tip for a few seconds and cut the inoculation loop with a scissor, leaving one-third of the loop inside the culture tube. ***If***

the agar plate is cold, use cold media during inoculation. If the plate is at 37 ℃, pre-warm each culture tube to the same temperature for ~20 minutes.

Bacterial Strain	LB Volume
Top 10	5 mL
DH10Bac	10 mL

d) Incubate the culture for ~12–16 hours (preferably 16 hours) in an orbital shaker incubator at 37 °C, 225 r.p.m. The samples should look turbid, indicating bacterial growth.

e) Decontaminate all materials and working area with 70% ethanol. Carefully dispose the bleach solution into the proper waste container and discard the materials in a biological waste (can with red bag).

f) Sanitize the working area and materials with 70% ethanol. Return any material and equipment to their proper place.

Glycerol Stock Procedure:

a) Decontaminate the working area and materials with 70% ethanol before placing anything on the bench.

b) Prepare the waste container with bleach for decontamination. ***Any material in contact with bacteria must be decontaminated in this container for at least 30 minutes***.

c) Organize and label the appropriate amount of cryovials to be used during the process. Add 300 μL of 50% glycerol to each cryovial.
   - Each cryovial must be labeled with the protein of interest, vector, sample number (e.g. #1), investigator's last name, cell strain, and date.

d) Add 2 mL of fresh LB media containing the appropriate antibiotic and chemicals into a 7–14 mL culture tube and pre-warm the tube in an incubator with an orbital shaker (37 °C, 225 r.p.m.) for ~20 minutes. ***Inoculating cold-temperature media could affect the growth rate of the bacteria***.

e) Remove the inoculation loop from the culture tube and throw it into the waste container with bleach.

f) Inoculate the 2 mL of LB media with 50 μL of the overnight bacterial culture and check the cap is loose but fixed to the tube to let oxygen reach the media. If you are using a screw cap, do not screw the cap and use tape to hold the lid. Place the bacterial culture in the orbital shaker for incubation. ***The remaining overnight media will be used for plasmid purification using the appropriate miniprep kit (silica or anion-exchange columns).***

g) Check the absorbance of the culture every hour at 600 nm. When the absorbance reaches an optical density (OD_{600}) of ~0.5–0.7, mix 700 μL of the culture with the 300 μL of 50% glycerol in the cryovial and vortex briefly.
   - ***Please refers to the Spectrophotometer Manual or S.O.P. for further instructions***.
   - ***Use pre-warmed (37 ℃) LB media with the appropriate antibiotic and chemicals as blank.***
   - ***When measuring the absorbance of several culture tubes at the same time, do not clean the cuvette with RO Water between measurements; just be sure all the media was removed from the cuvette. Otherwise, clean the cuvette between measurements.***

h) Incubate the cryovial for 30 minutes at room temperature to allow the cells to incorporate glycerol.

i) Store the cryovial at –80 °C.

j) Decontaminate all materials and working area with 70% ethanol. Carefully discard the bleach solution into the proper waste container and discard the materials in a biological waste (can with red bag).

k) Be sure all equipment was shut down: orbital shaker and spectrophotometer. Return any material and equipment to their proper place. The pipettes must be returned to their maximal volume.