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SOP

Standard Operating Procedure

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Title:

PRESERVATION OF BACTERIA USING COMMERCIAL CRYOPRESERVATION SYSTEMS

References:

Reviewed & approved by: All ASIARESIST partners

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PRESERVATION OF BACTERIA USING COMMERCIAL CRYOPRESERVATION SYSTEMS

A. PURPOSE & PRINCIPLE

The purpose of this SOP is to describe the procedure for long time storage of bacterial cultures. Organisms should be stored at low temperatures utilizing a mechanical technique that results in a minimal disturbance of the material. Cryopreservation systems such as Microbank™ and Protect™ contain cryovials of app. 25 beads in a cryopreservative. The porous nature of the beads allows adherence of the bacteria onto the bead surface. The cryovials are stored at -70°C, and a fresh culture can readily be obtained by inoculating one single bead onto the approperiate medium.

B. METHOD DESCRIPTION

All handling in this SOP should be performed under ASEPTIC conditions!

I. Bacterial cultivation and material preparations (DAY 1)

- The organism to be stored should be cultivated on a suitable agar medium under optimal incubation conditions to obtain a fresh overnight grown culture. As standard conditions, growth on Iso-Sensitest Agar (ISA; Oxoid) supplemented with 35 ppm chloramphenicol (CHL) at 28°C under aerobic atmosphere are recommended. **The inclusion of CHL in the medium mimics the antibiotic pressure that may be necessary to maintain the genomic elements (e.g. plasmids) responsible for CHL resistance**. Streak out the pure culture on an ISA+CHL plate in a way that distinct colonies will be obtained.
- Prepare sterile cotton swabs and Pasteur pipettes

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II. Inoculation of cryopreservation vials (DAY 2)

- Label the cryovial with strain or isolate designation and date of preparation using a water-resisting marker. Prepare two cryovials per isolate, preferably with two different colours of the screw cap (e.g. green and red).
- Harvest app. half of a full grown plate with a sterile cotton swab and make a rich suspension by dipping in the cryopreservative solution so that all beads come in contact with the cell suspension. Once the bacteria are deposited, close the cryotube and mix well by inverting 10 times. Repeat for the second vial.
- One vial will be the 'working' vial (e.g. green) wheras the other vial will function as the 'backup' vial (e.g. red). Remove all supernatans using a sterile Pasteur pipette from both working and backup vials. The latter vial will only be used if the 'working' vial is nearly running out of beads or in case of bad recovery.
- Quickly freeze both vials at -70°C.

III. Cryopreservation check (AT LEAST 48H AFTER FREEZING)

- Using a sterile (= flamed) wire, one bead is removed from the cryovial and rubbed repeatedly back and forth over the surface of an ISA+CHL plate to obtain a thick inoculation line. Using a conventional streaking loop, the inoculation line is further streaked out in a way that different colonies are expected to arise. The streak culture is incubated aerobically at 28°C.
- Check growth and purity of the plate culture after 24h to estimate the recovery of the cryovial culture

IV. Harmonization of method between collaborating laboratories (START OF STUDY)

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- In order to correlate the recovery of bacterial isolates in different laboratories, it is necessary to harmonize the inter-laboratory logistics. For this purpose, a set of taxonomically well-defined strains should be circulated between laboratories in the form of agar subcultures. For each strain of the harmonization set, the following steps should be carried out on ISA medium (without CHL!)
    - Prepare a cryovial culture from the received slant culture and store at -70°C for at least 48h
    - Subculture the stored strain from the prepared cryovial and check recovery and purity
    - Prepare a small agar slant for postal transfer to the core collection lab

  Taxonomic characterization (fatty acid analysis) will be used to compare the returned subculture with the original culture.

C. REFERENCES

- White and Sand (1985) Medical Laboratory Sciences 42:289-290 (UK)

D. MATERIALS

- **Iso-Sensitest Agar (ISA)**
  # CM 471 (Oxoid, Basingstoke, UK)

  ISA medium should be prepared with distilled or freshly deionised water. Make sure that each bottle contains a magnetic stirrer. A known volume (app. 500 mL) of sterilized ISA medium should be cooled down to 50-55°C. Poured plates may be stored for up to 3 days in air-tight plastic bags at 2-8 °C. Immediately prior to inoculation media should be moist but free of droplets, which should not be present on either the agar surface nor on the petri dish lids. If necessary plates may be dried by incubation at 30-37 °C or in a laminar flow cabinet for a maximum of 30 min. A representative sample of each batch of plates should be examined for sterility by incubation at 28 °C for 72 h.

- **Iso-Sensitest Agar supplemented with chloramphenicol (ISA+CHL)**
  # CM 471 (Oxoid, Basingstoke, UK)

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ISA medium should be prepared with distilled or freshly deionised water. Make sure that each bottle contains a magnetic stirrer. A known volume (app. 500 mL) of sterilized ISA medium should be cooled down to 50-55°C. Subsequently, the chloramphenicol stock solution (100x concentrated) is added and well mixed by magnetic stirring. Try to avoid the creation of air bubbles when homogenizing the selective medium.
Poured plates may be stored for up to 3 days in air-tight plastic bags at 2-8 °C. Immediately prior to inoculation media should be moist but free of droplets, which should not be present on either the agar surface nor on the petri dish lids. If necessary plates may be dried by incubation at 30-37 °C or in a laminar flow cabinet for a maximum of 30 min. A representative sample of each batch of plates should be examined for sterility by incubation at 28 °C for 72 h.

- **Chloramphenicol (CHL) stock solution**
  Preferably, all participating labs should use the same brand of chloramphenicol. In the current project, all partners have agreed to purchase CHL from Oxoid in the form of Selective Supplement vials containing 50 mg CHL each (# SR0078, Oxoid).
  The CHL stock solution needs to prepared with ethanol according to the manufacturer's instructions (http://www.oxoid.com/uk/index.asp?mpage=iproductdetail&pre=SR0078&l=EN&x=).
  Consequently, it is not necessary to filter-sterilize the solution as the chance that bacteria can survive in ethanol is negligible. The stock solution should be stored in the refrigerator until addition to the ISA medium.
  For selective isolation of CHL-resistant bacteria, CHL is added to the isolation medium in an empirically defined breakpoint concentration. Prepare the CHL stock solution in a concentration that is 100 times more concentrated than the breakpoint concentration to keep stock solution volumes as small as possible. In this way, a selective medium solution can be obtained by adding 5 mL 100x stock to a bottle of 500 mL ISA medium (i.e. 1/100 dilution).

- **Reference strains in harmonization set**

  Escherichia coli LMG 8223

  Aeromonas hydrophila LMG 2844

  Stenotrophomonas maltophilia LMG 11098

  Vibrio harveyi LMG 4044

  Acinetobacter junii LMG 10577

  Salmonella enteritidis LMG 10395

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- **Microbank™ vials**
  Shelf packs of 50 vials (PRO-LAB Diagnostics, Neston, Wirral, UK)