Storage of mycobacterial strains

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1. Introduction

The storage and maintenance of mycobacterial reference strains and clinical isolates is an important part of good laboratory practice in a mycobacteria laboratory. The storage of reference- and control strains facilitates a reliable control on intra- and inter-test reproducibility ***(1,2)***. Furthermore, to study various aspects of the epidemiology of tuberculosis it is important to be able to study serial isolates of individual patients ***(3)*** or of patients associated with particular place- or time factors ***(4)***.

It is possible to maintain mycobacterial cultures by reculturing on solid medium, like Löwenstein-Jensen medium. There are, however, many examples of marked genetic re-arrangements in mycobacterial strains during *in vitro* culturing ***(5,6)***. It is therefore of the utmost importance to preserve reference strains and clinical isolates under circumstances with the lowest metabolic activity.

Previously, lyophilization was the most frequently used approach to preserve cultures. Because the viability of the lyophilizated bacteria drops dramatically within a few years, storage of cultures in –70°C freezers is increasingly applied. In the Netherlands a 15 years-experience with latter method for storage has shown that this method is highly efficient to maintain the viability of mycobacterial cultures.

This chapter describes how to establish and maintain a mycobacterial strain collection and how strains can be stored at -70°C in glycerol.

2. Materials

2.1 Establishing a mycobacterial strain collection

1. Sticker machine (e.g. the Brady LS2000, WH Brady N.V., Zele, Belgium) (*see* **Note 1**).
2. Culture media tubes containing either solid- or liquid medium (*see* **Note 2**).
3. Solid medium: Löwenstein-Jensen medium or Löwenstein-Jensen medium supplemented with pyruvate ***(7)*** (*see* **Note 2**).
4. Liquid medium: 5 mL Middlebrook 7H9 medium ***(7)*** (*see* **Note 2**).
5. Öses.
6. Pipettes with a cloth (2 mL).
7. Incubator 30°C, 35,5°C, and 42°C (*see* **Note 3** and **Table**).
8. Freezer, -70°C (*see* **Note 4**).

Table
Optimum temperatures and media to grow mycobacteria

Species	Temperature (°C)
M. tuberculosis complex	35.5
M. avium complex	30
M. malmoense	30
M. xenopi	45
other mycobacterial species	35.5

2.2 *Storing mycobacterial cultures in the –70°C freezer*

2.2.1 Preparation

1. *See* **Subheading 2.1., item 1**.
2. Cryovial tubes, with screw thread on the outside and a content of about 2 mL (e.g. Cryovials, 2 mL, Simport Plastic, Quebec, Canada)
3. Dry-ice-mixture; prepare the following mixture (*see* **Note 5**):

   100 mL crushed dry-ice;

   100 mL 80% alcohol;

   50 mL tap water.

   Mix well and store in a tempex box.

2.2.2. Storing solid mycobacterial cultures in the –70°C freezer

1. Storage medium; prepare the following solution:

   3 g Tryptone Soya Broth (Oxoid ltd., Basingstoke, UK);

   20 mL glycerol (87%, Merck, p.a., Darmstadt, Germany);

   80 mL distilled water.

   Autoclave, store at 4°C for no longer than six months.

2. *See* **Subheading 2.1., items 5, 6, and 8**.

2.2.3. Storing liquid mycobacterial cultures in the –70°C freezer

1. Concentrated storage medium; prepare the following solution:

   6 g Tryptone Soya Broth (Oxoid ltd.);

   40 mL glycerol (87%, Merck, p.a.);

   80 mL distilled water.

   Autoclave, store at 4°C for no longer than six months.

2. *See* **Subheading 2.1., items 6 and 8**.

2.2.4. Storing a large batch of a strain

1. *See* **Subheadings 2.1., 2.2.1., and 2.2.2.**.

2.3 Reculturing strains from the –70°C freezer

1. *See* **Subheading 2.1.,** and **Subheading 2.2.1., item 3**.

3. Methods

Caution: The species that may be pathogenic to humans should always be handled in the appropriate containment facilities.

3.1 Establishing a mycobacterial strain collection

1. Label each mycobacterial isolate that arrives at the laboratory with a unique strain number (*see* **Note 1** and **6**).
2. Label the patient data or other information belonging to that new isolate with the same strain number and enter it in a strain collection database (*see* **Note 7**).
3. Label at least two fresh culture media tubes for each new isolate and culture them with the appropriate isolate (*see* **Notes 1, 2** and **8**).
4. Incubate the cultures at the appropriate temperature (*see* **Note 3** and **Table**).
5. Store each strain in the -70°C freezer as described below.

3.2 Storing mycobacterial cultures in the -70°C freezer

Three methods to store mycobacterial strains in the -70 C freezer are described, one for mycobacteria grown on solid media, one for liquid mycobacterial cultures, and one for storing a large batch of a certain strain (*see* **Notes 2, 4** and **9**).

3.2.1. Preparation

1. Select a well-grown (non-contaminated) solid or liquid culture of each the mycobacterial strains that should be stored (*see* **Note 2**).
2. Label two cryovial tubes, both on the top and the side, for each strain that is to be stored, with the strain numbers of the respective strains (*see* **Note 10**).
3. Prepare a fresh dry-ice-mixture as described in **Subheading 2.2.1., item 3**.

3.2.2. Storing solid mycobacterial cultures in the -70°C freezer

1. Add 2 mL storage-medium to the slope with a pipette.
2. Resuspend the mycobacteria with an öse (*see* **Note 11**).
3. Pipette the bacterial suspension from the slope and divide it over the two cryovial tubes labelled with the strain number of the respective strain.
4. Put the cryovial tubes containing the suspension as quickly as possible on the dry-ice-mixture (*see* **Note 12**).

5. Store at -70°C after the suspensions are completely frozen (*see* **Note 4**).
6. Register the storage of the cultures on an appropriate form or in the strain collection database.

3.2.3. Storing liquid mycobacterial cultures in the –70°C freezer

1. Add 1 mL concentrated storage medium to a cryovial tube labelled with the strain number of the strain that is to be frozen.
2. Collect the sediment (total volume 1 mL) of the mycobacterial culture that is to be frozen with a pipette and add it to the 1 mL storage medium.
3. Homogenise the bacterial suspension by carefully pippetting a few times up and down (*see* **Note 11**).
4. Pipette 1 mL suspension to the other labelled cryovial tube.
5. Continue with **Subheading 3.2.2., items 4 to 6**.

3.2.4. Storing a large batch of a strain (*see* **Notes 13** and **14**)

1. Culture the strain on twelve labelled culture medium tubes and incubate them at the appropriate temperature until they are well-grown (*see* **Notes 2** and **3,** and **Table**).
2. Label 24 cryovial tubes with the strain number of the respective strain, both on the side and the top (*see* **Note 10**).
3. Transfer as many bacteria as possible to 24 mL storage medium (*see* **Note 15**).
4. Homogenise the suspension by swirling.
5. Distribute 1 mL volumes of the suspension to the labelled cryovial tubes.
6. Continue with **Subheading 3.2.2., items 4 to 6**.
7. Perform a quality control on the batch by checking the growth and the biochemical and/or molecular characteristics of the respective strain.

3.3 Reculturing strains from the –70°C freezer (see Note 16)

1. Label two culture media tubes with the strain number of the strains that are to be taken from the freezer (*see* **Note 2**).
2. Prepare a fresh dry-ice mixture as described in **Subheading 2.2.1., item 3**.
3. Take the cryovial tube from the freezer and keep it on the dry-ice-mixture.
4. Scrape some frozen suspension from the tube with an öse, while keeping the suspension frozen, and culture this on the two labelled culture media tubes (*see* **Notes 2** and **17**).
5. Place the cryovial tube back into the freezer as soon as possible.
6. Incubate the cultured tubes at the appropriate temperature (*see* **Note 3** and **Table**).

4. Notes

1. To label the tubes it is convenient to use a sophisticated sticker machine (e.g. Brady LS2000, WH Brady N.V.). Such a machine can prepare several stickers with the same number at once, and in addition prepare series of stickers with incremental numbers or date. Alternatively a more basic sticker machine can be used (a supermarket stickermachine, e.g. Meto 826, Esselte Meto bv, Nieuwegein, The Netherlands).
2. For various (mainly practical) reasons it may be more convenient to use either a solid or a liquid culture. Generally, you can choose the culture medium you are accustomed to. The procedure to store liquid media can be executed faster and is therefore recommended if many strains are to be stored. Solid media have the advantage that it is more easy to see whether the culture is contaminated or not, whereas this is difficult to see when liquid media are used.
3. Different mycobacterial species require different optimal growth conditions. See the **Table** for the optimal incubation temperatures for the various mycobacterial species.
4. It is recommended to prepare the culture collection in duple. Therefore, use two -70°C freezers, and store the two cryovial tubes of each strain in different freezers. Preferably these two freezers should be at different locations, and depending on different power supplies. In this way, in case of an accident (e.g. fire in the laboratory, or long periods of time without electricity) the culture collection is still maintained. For practical reasons, one freezer should be kept in the laboratory.
5. The dry-ice-mixture is well-prepared when it looks mushy. In this case the cryovial tube has immediate contact with the ice everywhere, and therefore freezes most efficiently.
6. Each strain should have an unique strain number. The patient data and the patient's isolate should be linked to this number. Preferably this strain number should be used for all the procedures in the laboratory in which the culture is handled. The advantage of a numbering code consisting of a year and a serial number is that one more or less knows when the sample was received, but the disadvantage is that writing errors may occur more easily.
7. It is recommended to use a strain collection database (e.g. Access, Microsoft Co., Redmond, Washington, USA) or a dedicated laboratory information management system to store all the patient- and experimental data of the patient's isolate in, simultaneously linking the patient data to an unique strain number.
8. It is recommended to sub-culture each new isolate on at least two culture media tubes. One tube can then be used for storing the isolate in the -70°C freezer, the other tube (or tubes) for laboratory procedures. In addition, the tube(s) used for laboratory procedures can be kept at room temperature

for about three to six months. In case additional tests are required using this tube may be quicker than reculturing the strain from the -70°C freezer.

9. It is recommended to prepare a large batch of reference- or other strains that are taken from the freezer more frequently. A less time consuming protocol to store many vials of a such strains is described here.
10. Use stickers that do not come off after thawing, or cover the sticker with tape. If a culture collection is maintained in duple it may be convenient to label the two cryovial tubes with different colours. For this purpose plastic labels that fit into the top of the lid of the cryovial tubes are commercially available.
11. Resuspend the mycobacteria carefully, avoid the forming of aerosoles.
12. The suspensions should be frozen as quickly as possible in order to keep the storage medium homogeneous and to damage the bacteria as little as possible.
13. When a large batch of a strain is to be stored, it is recommended to grow the strain on solid medium because now it is especially important to know that the culture is pure (not contaminated). This protocol describes the use of 12 grown cultures. Of course, if desired, this number can either be increased or decreased. If so, then the number of cryovial tubes and the volume to suspend the bacteria in should be adjusted accordingly.
14. It is also possible to use a liquid culture, but besides the disadvantage of the worse recognition of contaminations, it is also difficult to estimate the volume of storage medium that is to be used to dissolve the bacteria in. If a liquid culture is preferred, then use the following protocol: (i) grow the strain on e.g. 50 or 100 mL liquid medium (ii) When the culture is well-grown, centrifuge at 1200*xg* for 15 minutes, and discard the supernatant (iii) dissolve the pellet in 5 to 10 mL storage medium, depending on the size of the pellet. (iiii) continue with **Subheading 3.2.4., item 4**.
15. The volume of storage medium and the number of cryovial tubes to distribute the homogenised suspension in can be increased if the growth on the culture tubes was abundant.
16. It may be useful to reculture certain reference strains regularly (e.g. each month) from the -70°C freezer, so that there is always a fresh culture of that strain available.
17. If a cryovial tube from the collection is almost empty or defrosted, then subculture a new culture medium tube and repeat the procedure described in **Subheading 3.2**, to store the strain again. Register the replacement on an appropriate from or in the strain collection database.

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