STANDARD OPERATING PROCEDURE for:

Department of Applied Technology, Institute of Applied Sciences and Technology, National Centre of Research and Technology, Burkina Faso; **Food Research Institute**, Council for Scientific and Industrial Research, Ghana; **University of Development Studies**, **Department of Applied Biology**, Ghana; **University of Abomey-Calavi**, **Faculty of Agricultural Science**, Benin, under the GreenGrowth Project

Handling, maintenance and preservation of microbial isolates in culture-collection

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Contents

1. Scope and objective

This procedure is in its original form made as a part of the project Preserving African Food Microorganisms for Green Growth (DFC No. 13-04KU).

The purpose of this procedure is to describe the handling, maintenance and preservation of microbial isolates, in order to ensure their identification, traceability and safety and to preserve their technological properties, in the culture-collections under the GreenGrowth project located at:

- Department of Applied Technology, Institute of Applied Sciences and Technology, National Centre of Research and Technology, Burkina Faso
- Food Research Institute, Council for Scientific and Industrial Research, Ghana
- University of Abomey-Calavi, Faculty of Agricultural Science, Benin

This procedure applies to all isolates currently available at the microbiology laboratories of partner institutions in the GreenGrowth project, as well as to new isolates that will be generated by new research/food quality control activities during the GreenGrowth project.

This procedure is based on the World Federation for Culture Collection Guidelines for the establishment and operation of culture-collections of cultures of microorganisms (WFCC executive board, 2010)[1] and on the online resources from Common Access to Biological Resources and Information (CABRI) Guidelines; Laboratory guidelines for microorganisms[2].

Furthermore, this procedure is developed to fulfil the requirements for documentation as determined in the Rio Convention, Convention on Biological Diversity (1992)[3]. Hence, in terms of the Rio Convention on Biological Diversity this procedure aims at implementing instructions for preservation of components of biological diversity and instructions to monitor, through sampling and other techniques, the components of biological diversity. Also, this procedure aims at implementing a system to: "Maintain and organise, by any mechanism data. derived from identification and monitoring activities […]" (article 7c, p. 5)[3]. This procedure is based on: "*Ex-situ* conservation of components of biological diversity […] in the country of origin." (article 9a, p. 7)[3], with backup conservation in a nearby country. The aim is to: "Establish and maintain facilities for *ex-situ* conservation of and research on […] microorganisms […] in the country of origin of genetic resources." (article 9b, p. 7)[3]. Finally, it is the anticipation that this procedure can help to: "Establish and maintain programmes for scientific and technical education and training in measures for the identification, conservation and sustainable

References:

[1] WFCC executive board (2010). World Federation for Culture Collection Guidelines for the establishment and operation of culture collections of cultures of microorganisms, 3rd edition, ISBN 92 9109 043 3. http://www.wfcc.info/guidelines/

[2] http://www.cabri.org/guidelines/micro-organisms/MCover1.html

[3] United Nations, Rio Convention, Convention on Biological Diversity.

use of biological diversity and its components and provide support for such education and training for the specific needs of developing countries." (article 12a, p. 8)[3].

Another tentative standard operating procedure for the sampling of microorganisms from fermented food products and raw materials used for the processing of the fermented foods, including identification of the isolated microorganisms has additionally been developed under the GreenGrowth project with the title: "Tentative standard operating procedure; Sampling and identification of microbial isolates from food products" [SOP-GG-02-00], which is being referred to in this procedure where applicable.

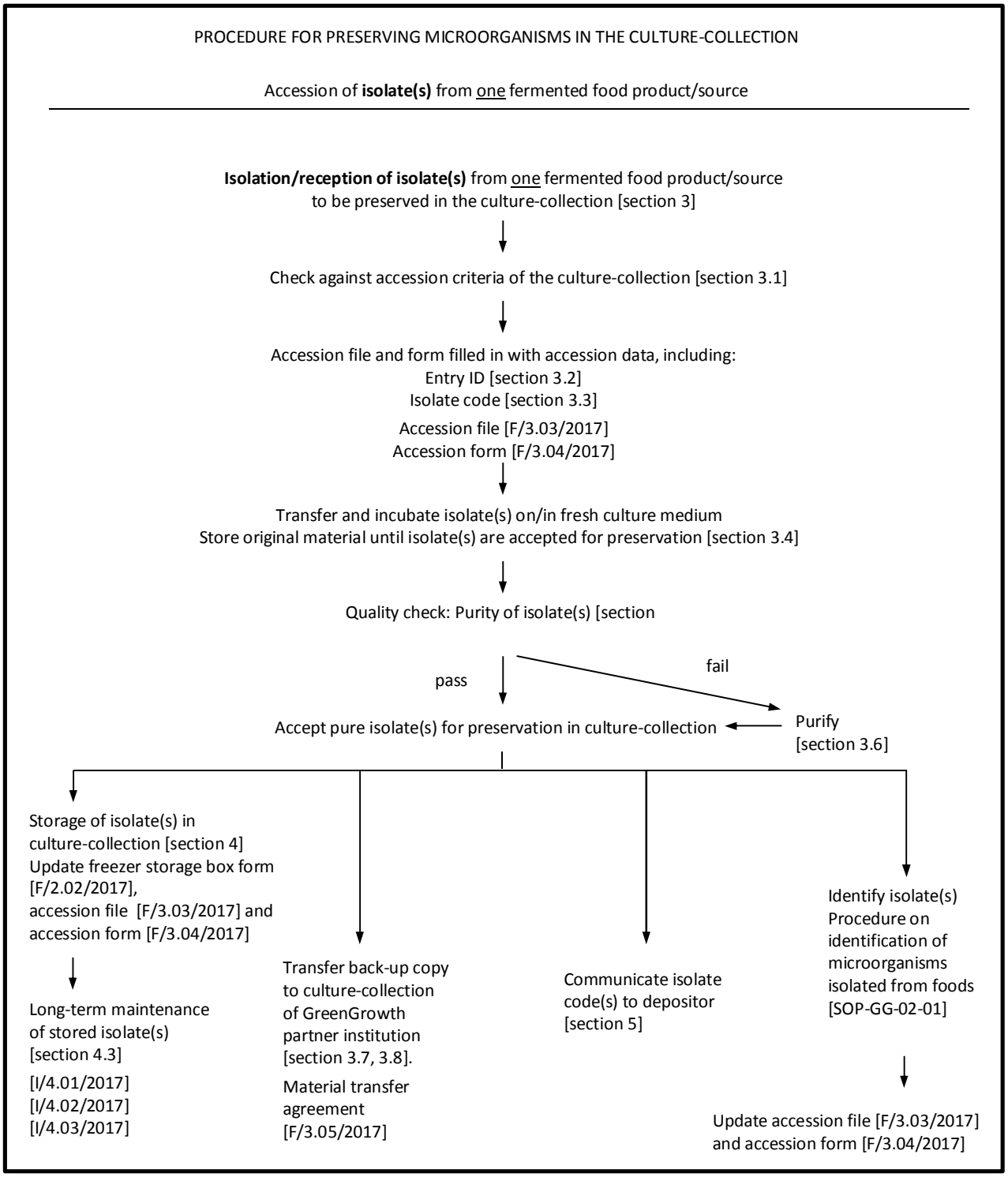
Financial support provided by Danida, Ministry of Foreign Affairs, Denmark, through the GreenGrowth project is gratefully acknowledged.

This procedure has the document ID: SOP-GG-01-00. The procedure should be reviewed for updating every two years.

Reference:
[3] United Nations, Rio Convention, Convention on Biological Diversity.

PROCEDURE FOR PRESERVING MICROORGANISMS IN THE CULTURE-COLLECTION

Accession of **isolate(s)** from one fermented food product/source



2. General freezer procedures

2.1 Installation of freezers and power back-up

The -80°C freezer should be placed in a chilled room protected from high ambient temperature. The freezer should be locked and the keys stored with only two staff members, appointed as managers of the culture-collection. The freezer temperature is recorded twice a day (upon arrival of the responsible staff and when leaving), using the freezer temperature control form [F/2.01/2017]. To avoid any power cuts, power back-ups, should be installed. Opening of the freezers should be minimized. Microorganisms deposited in the freezers should be kept in the freezers and only taken out for distribution to persons requesting isolate(s) at the beginning of experiments and for long-term maintenance procedures.

2.2 Allocation of responsibilities, including key responsible persons

Among the staff in the partner institutions a manager (research scientist) and an assistant manager (technician) should be appointed, who will be responsible for managing the culture-collection, and ensuring that this procedure [SOP-GG-01-00] is being implemented and followed. As stated in 2.1, the keys for the freezer are handled by the appointed culture-collection managers. Only the appointed culture-collection managers have the authority to enter the freezer and handle the isolates stored in the culture-collection.

2.3 Labelling of the culture-collection facility (labelling of freezer, shelves, racks, boxes and box positions)

Proper labelling of the culture-collection storage facility is important to ensure traceability of stored isolates. Freezer arrangement is presented in [A/01/2017].

Numbers should be assigned for [F/2.02/2017]:

- Freezers
- Shelves
- Racks
- Boxes
- Positions in the box (row letter and column number)

Example: F1S1R1B1A1 (i.e. freezer 1, shelf 1, rack 1, box 1, position; row A, column 1)

NOTE: it is important to write the box number reference on two pieces of waterproof paper, where the first one is taped on the outside of the box and the second one is placed in the interior of the box.

For each box kept in the freezer, a freezer storage box form is assigned [F/2.02/2017], in which the code of each isolate in the box is recorded. Upon storage of isolates in the culture-collection the freezer no., shelf no., rack no. and box no. and position in the box is noted in the electronic accession file [F/3.03/2017] and the print

out accession form [F/3.04/2017]. Storage freezing boxes with isolates should always be stored in the freezer in horizontal position with the lid up.

2.4 Overall procedure for handling and maintenance of microbial cultures

A flow diagram of the overall procedure for handling and maintenance of microbial cultures is presented in Fig. 1 [p. 3]. Reference to the relevant forms and instructions for each step in the procedure is indicated in Fig. 1.

3. Accession of isolates in culture-collection

All parcels arriving at the GreenGrowth culture-collections containing microbial isolates are handled by responsible person(s) for unpacking received biological specimens. Staff members responsible for unpacking of biological specimens should be aware of the potential hazard involved. Packages containing unknown cultures or cultures of microorganisms classified with risk group/class 2 or higher [A/02/2017] should be unpacked in a laminar air-flow cabinet. Broken or leaking containers should be handled with great care. Disinfection agents should always be available.

Named cultures belonging to risk groups not allowed to be preserved in the culture-collection should be immediately placed in an autoclave and heat sterilized.

The different steps in the accession of microorganisms in the GreenGrowth culture-collections, which is to be followed for all isolates to be preserved in the culture-collections is described in the flow diagram Fig. 1 [p.3].

3.1 Accession criteria

GreenGrowth culture-collections accept isolates of bacteria and yeasts, as long as they are within of risk groups 1 and 2 according to the World Health Organization classification system [A/02/2017], and when they additionally comply with at least one of the following criteria:

- Isolates from fermented foods, raw materials used for fermentation, final fermented food products
- Reference strains (e.g. type strains)
- Isolates that are used in research projects under the GreenGrowth project

Isolates accepted for deposit must be pure cultures or defined mixed cultures. Additionally, it must be possible to preserve the deposited material by freezing without significant change to the microorganisms.

3.2 Accession form and accession file

Isolates entering the culture-collection must be registered in two separate ways:

1. An electronic accession file [F/3.03/2017] (a continuously updated electronic file containing information on all isolates in the culture-collection)
2. A print out accession form [F/3.04/2017] (a separate form for each isolate, stored in a printed version kept in a folder)

Entry ID numbers (in Roman numeral, i.e. 01,02,03,…10,11,…100,101,…etc.) are assigned continuously to isolates entering the culture-collection. If multiple isolates originating from one fermented food product are to be preserved in the culture-collection (e.g. isolates from raw materials used for the fermentation, isolates sampled during the fermentation of the food product and isolates from final products), they are all assigned

the same entry ID to link them together in the electronic accession file and print out accession form, but with separate isolate codes to differentiate all the isolates originating from the product/source.

The following information, on each isolate to be deposited in the culture-collection, should to be obtained and filled in the electronic accession file [F/3.03/2017] and in the print out accession form [F/3.04/2017], when applicable:

- Entry ID [section 3.2]
- Isolate code [section 3.3]
- Freezer location (freezer no., shelf no., rack no., box no., position in the box) [section2.3]
- Date of arrival
- Date of deposit
- Name of depositor
- Country for isolation
- Name of product
- Name of production site
- Sampling report ID [SOP-GG-02-00, section 2.2]
- Sample ID [SOP-GG-02-00, section 2.3]
- Name of person collecting the samples [SOP-GG-02-00, section 2.2]
- Name of person isolating the microorganisms [SOP-GG-02-00, section 2.2]
- Name of identified species, subspecies or strain(s) [SOP-GG-02-00, section 5]
- Identification method used [SOP-GG-02-00, section 5]
- Name of person identifying the isolate
- Source of the isolate if the isolate originate from another collection
- Proposed date for revivification [section 4.3]
- Culture-collection for back-up storage and other places for deposition of isolate including isolate code, if applicable [section 3.7]
- Optimal growth conditions (media, temperature and aerobic/anaerobic etc.)
- Technological properties (if known) [SOP-GG-02-00, section 5.1]
- Safety information

3.3 Isolate code labelling

Traceability of isolates is assured by assigning **isolate codes** as described in the following. **The isolate code is permanently bound to a specific isolate and is never changed** although the scientific name of the isolate may change.

In the electronic accession file [F/3.03/2017], the print out accession form [A/3.04/2017], the freezer storage form [F/2.02/2017] and on cryotubes containing the isolate, labelling is performed using a code comprising:

- The initials of the institute; DTA (Départment Technologie Alimentaire, Burkina Faso), FRI (Food Research Institute, Ghana), UDS (University for Development Studies, Ghana), UAC (University of Abomey-Calavi, Benin)
- Entry ID; 01,02,03,…10,11,… etc. [section 3.2]
- Number of microbial isolate; 01,02,03,…10,11,… etc.
- Copy number of the isolate; 1,2,3,4,5 [section 4.1]

Example of **isolate code**: DTA-01-01-4 (i.e. an isolate from DTA, entry ID 01, isolate number 01, copy 4 (working cryotube)).

3.4 Transfer and incubation of isolate(s) in fresh culture medium

After reception of isolate(s) for deposit in the culture-collection, the isolate is transferred and incubated in fresh liquid media. Media composition and preparation are described in [A/03/2017].

Original culture material of a deposited isolate should be maintained, in the fridge at 5°C ± 2°C, at least until after positive controls of permanent preservation methods is obtained. Great care must be taken to ensure as far as possible the originality of the cultures. Therefore, cultures deposited are preserved with a minimum number of transfers.

Procedure:

- Transfer 1 colony using a sterile loop or 100 µL of liquid culture to 10 mL of sterile appropriate broth in sterile tube, Table 1 [p. 9]
- Incubate at appropriate temperature, Table 1 [p. 9]
- After growth, streak the isolate onto ¼ of the surface of the appropriate solid substrate as shown in Fig. 2 [p. 9]
- Incubate the petri dish for the appropriate time, temperature and condition, Table 1 [p. 9]

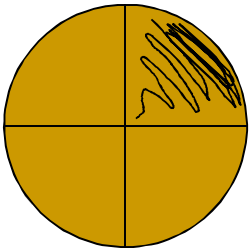


Fig. 2. Schematic presentation of procedure for purification test of an isolate. An isolate grown in the appropriate broth is streaked onto ¼ of the surface of the appropriate agar plate, to obtain single colonies on the agar plate upon incubation.

Table 1: Media and incubation conditions, temperature and time for the different groups of microorganisms. Media composition and preparation methods [A/03/2017].

Microorganism	Media	Condition on agar	Temperature	Incubation time (broth)	Incubation time (agar)
LAB	MRS, pH 6.2	Anaerobic	30°C	24h-48h	3-5 days
Lactobacillus spp.	MRS, pH 5.4/6.2	Anaerobic	30°C	24h-48h	3 days
Lactococci and streptococci	M17 + 0.5% (w/v) lactose	Aerobic	30°C or 37°C	24h-48h	3 days
Bacillus spp.	Plate count or Nutrient	Aerobic	30°C	24h-48h	1-3 days
Yeasts	MYGP	Aerobic	25°C or 30°C	24h-48h	3-5 days

3.5 Quality check of isolates

For isolates to be deposited in the culture-collection the viability and purity is tested by subculturing the original culture received.

The following tests are considered as a minimum for preliminary quality check of new deposits:

- Viability: growth in liquid media [section 3.4]
- Purity: isolate(s) from section 3.4, streaked on appropriate agar plates is checked for purity, Table 1 [p. 9] and Fig. 2 [p. 9]. Pure isolates show uniform colony characteristics and no contamination occur on the plate
- Macroscopical characterisation: colony morphology, colony colour
- Microscopical characterisation: cell shape, cell size, cell motility, sporulation (yeasts), spore formation (*Bacillus* spp.), using phase contrast microscopy with 100X objectives for bacteria and 40X objectives for yeasts

If the isolate is viable and pure the microorganism is accepted for deposit and preserved as described in [section 4] and [I/4.01,02,03/2017]. If the isolate fails the purity test, purification is performed as described in [section 3.6].

3.6 Purification of isolates that failed the purity quality check

Impure isolates must be purified before being deposited in the culture-collection

Purification is performed as described in the following:

- Pick a single colony of the predominant colony type, based on colony shape and colour (from the purity plate, described in [section 3.4, 3.5]
- Transfer the selected colony to 10 mL of sterile appropriate broth in a sterile tube. Whirly mix and incubate for the appropriate time and temperature, Table 1 [p. 9]
- After incubation, streak the culture on appropriate solid substrate as shown in Fig. 2 [p. 9]
- Incubate the plates for the appropriate time, temperature and condition, Table 1 [p. 9]
- After incubation, one pure colony is streaked again on the surface of the appropriate solid substrate to confirm the purity of the isolate, as shown in Fig. 2 [p. 9]
- Remember to label purification plates and tubes with the isolate code [section 3.3]
- Pure isolates have uniform colony characteristics and are free of any contamination
- Upon successful purification, the isolate is accepted for deposit in the culture-collection and preserved as described in [section 4] and [I/4.01,02,03/2017]

3.7 Back-up copies of isolates

A back-up copy of all isolates, accepted for deposition in the culture-collection, is transferred to a culture-collection at another GreenGrowth partner institution. Materials transfer agreements [F/3.05/2017] are signed upon transfer and the back-up location is noted in the electronic accession file [F/3.03/2017] and the print out accession form [F/3.04/2017]. Transport of isolates should be carried out as described in [section 3.8].

3.8 Transport of isolates

To maximize survival of the microorganisms during transport, isolates should be inoculated on agar slants in cryotubes/eppendorf tubes, using the appropriate media of the microorganism, Table 1 [p. 9].

- Agar slant are prepared by adding 1 mL of appropriate agar to a tilted cryotube/eppendorf tube and let to solidify in the tube, Fig. 3 [p. 11]
- Cryotubes/eppendorf tubes are carefully marked with the isolate code
- For LAB the agar slant is inoculated by piercing the surface of the solidified agar slant with an isolate from plate or isolate material from overnight culture, using a sterile needle

- For *Bacillus* spp. and yeasts the agar slant is inoculated by streaking an isolate from plate or isolate material from overnight culture on the surface of the slant, using a sterile loop or needle
- The inoculated tubes are now ready for transport

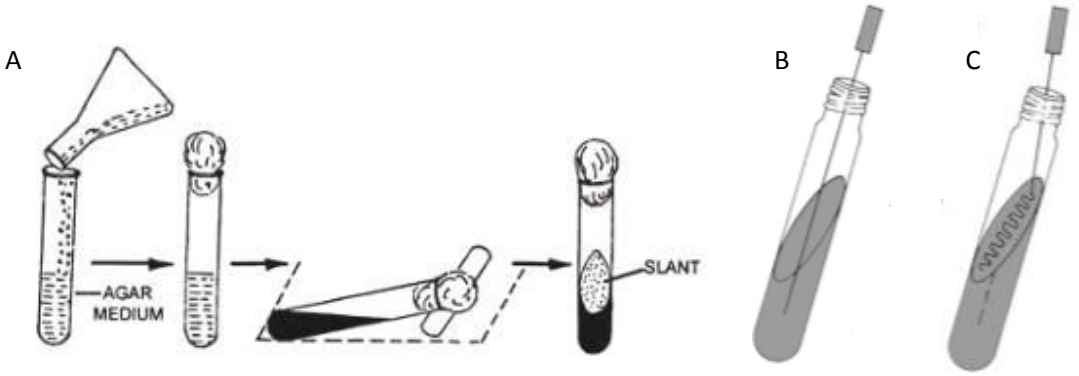


Fig 3. Illustration of (A) agar slant preparation, used for transport of isolates e.g. for back-up storage. (B) Illustration of inoculation of agar slant for LAB and (C) for *Bacillus* spp. and yeasts. Note: cryotubes/eppendorf tubes should be used for agar slant preparation instead of the tubes depicted in the illustration.

4. Preservation of isolates in the culture-collection, thawing and long-term maintenance of isolates

The following sections describe the procedures for preservation and maintenance of microorganisms in the culture-collection, i.e. freezing isolates [section 4.1], thawing isolates [section 4.2] and long-term maintenance of isolates [section 4.3].

- Any entry or exit of microbial isolates from the -80°C freezer must be done by the responsible scientist/technician in charge of the culture-collection
- For isolate(s) requested by a person, the responsible scientist/technician in charge streaks out the isolate(s) on appropriate solid substrate(s) and subsequent to incubation passes the isolate(s) on the plate(s) to the person/client who requested the isolate(s)

4.1 Freezing of isolates (entry of isolates to the -80°C freezer)

For isolates entering the -80°C freezer the procedures are:

- Pure isolates [section 3.5, 3.6] are prepared for freezing as described in instructions:
  - LAB [I/4.01/2017]
  - *Bacillus* spp. [I/4.02/2017]
  - Yeasts [I/4.03/2017]
- At least five copies of each isolate should be prepared in screw cryotubes, properly labelled with isolate code [labelling, see section 3.3].
  The five copies of the cryotubes are assigned as follows:
  - Copy 1: Back-up cryotube, which should be transferred to a GreenGrowth partner institution for back-up storage [F/3.05/2017] and only used if 3 is contaminated or unculturable
  - Copy 2: Cryotube used for revivification [section 4.3]
  - Copy 3: Cryotube for preparation of new working cryotubes. When copy 4 and 5 has been used, copy 3 is used to prepare three new copies of the isolate (copy 1, copy 2 and copy 3), as described in [I/4.01,02,03/2017]
  - Copy 4 and copy 5: Working cryotubes, which are used for experimental purposes or for clients. Optional, more working cryotube copies can be prepared, they are assigned copy 6,7,etc.
- Documentation of isolates are performed as described in [section 3] and the following forms/file are updated:
  - Freezer box storage form [F/2.02/2017]
  - Accession file [F/3.03/2017] (electronic)
  - Accession form [F/3.04/2017] (print out)

4.2 Thawing isolates (exit of microbial isolates from the -80°C freezer)

Thawing of an isolate is only carried out when a person/client is requesting the isolate. Only the appointed scientist/technician managing the culture-collection has the authority to carry out the procedure for thawing of isolates. In case of contamination in one of the working cryotubes, immediately discard the tube [section 4.1]. Remember to update the freezer box storage form [F/2.02/2017], the electronic accession file [F/3.03/2017] and the print out accession form [F/3.04/2017].

Thawing procedure:

- Only the working cryotube(s) for the ordered isolate(s) is taken out from the freezer and thawed at room temperature
- Streak out the isolate on the surface of the appropriate substrate, Table 1 [p. 9] or as listed in [P/2017/4.01,02,03]. In cases were multiple isolates are streaked out simultaneously, cryotube(s) must be handled carefully to avoid contamination or destruction of the microbial culture(s)
- After streaking, the cryotube(s) is discarded
- The plate is incubated for the appropriate time, temperature and condition, Table 1 [p. 9]
- Subsequent to incubation of the isolate(s) on plate(s), the plate(s) is passed on to the person requesting the isolate(s)
- Instructions for thawing of:
    - LAB [I/4.01/2017]
    - *Bacillus* spp. [I/4.02/2017]
    - Yeasts [I/4.03/2017]

4.3 Long-term maintenance of isolates stored in the -80°C freezer

Revivification can be performed every five years for individual isolates, which have been stored in a freezer with a stable temperature (i.e. has changed < ±20°C from the optimal -80°C) through a five year period. However, revivification of the individual isolates, which has been stored in a freezer with a temperature changing > ±20°C from the optimal -80°C during the deposit, revivification should be performed every second year.

Revivification is performed using copy 2 of the isolate as described in [section 4.1]. The revivified culture is replacing all copies of the isolates in the in-house freezer and the back-up freezer.
Instructions for revivification of:

- LAB [I/4.01/2017]
- *Bacillus* spp. [I/4.02/2017]
- Yeasts [I/4.03/2017]

A proposed date for the next revivification is recorded in the electronic accession file [F/3.03/2017] and in the print out accession form [F/3.04/2017].

The revivification includes:

- The revivification cryotube is removed from the freezer and the isolate streaked out on the surface of the appropriate solid substrate using a sterile loop and incubated for the appropriate time, temperature and conditions, Table 1 [p. 9] and as described in [I/4.01,02,03/2017]
- Control the streaked isolate for purity by examining colony morphology and colony colour of the isolate, which should be uniform and free from any contamination
- At least five new cryotube-cultures are prepared, properly labelled with isolate code [section 3.3]. The revivified cultures replace the cryotubes in the freezer [section 4.1]
- The revivified isolate cultures are prepared for freezing as described in instructions:
    - LAB [I/4.01/2017]
    - *Bacillus* spp. [I/4.02/2017]
    - Yeasts [I/4.03/2017]

5. Communicate isolate code(s) to depositor

Upon accepting isolates for deposit in the culture-collection, the isolate code(s) of the isolate(s) is communicated to the depositor. Below is an example of a standard letter:

Dear ..... ,

The following isolates(s) sent by you for deposit in the GreenGrowth culture-collection at [insert institution name] has/have been accepted for deposit on [insert date of acceptance] and assigned the following isolate code(s):

Isolate code Name of organism

Yours sincerely,

6. Enclosures

Forms

[F/2.01/2017] Temperature control
[F/2.02/2017] Freezer box storage form
[F/3.03/2017] Accession file for isolates in the culture-collection (electronic)
[F/3.04/2017] Accession form for isolates in the culture-collection (print out)

Instructions

[I/4.01/2017] Preservation and long-term maintenance of LAB in the culture-collection
[I/4.02/2017] Preservation and long-term maintenance of *Bacillus* spp. in the culture-collection
[I/4.03/2017] Preservation and long-term maintenance of yeasts in the culture-collection

Attachments

[A/01/2017] Freezer arrangement
[A/02/2017] World Health Organization –Classification of infective microorganisms by risk group
[A/03/2017] Media

NB: Enclosures are not included in this version.