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Short- and long-term storage of bacterial isolates

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FAO Reference Centre designated by Letter of Designation dated 5 November 2019
Food and Agriculture Organization of the United Nations

What are the advantages of bacterial preservation?

01

Preserving bacterial cells at the stage close to original isolation with no contamination until use.

02

Protecting against phenotypic drift due to genetic instability or selective pressures.

03

Creating a standard working stock for future experiments.

04

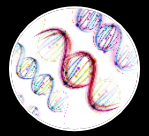
Reduction of time, effort, and materials needed to maintain bacterial strains not currently in use.

Effective bacterial storage

Ensure cell viability

Free from contamination

Retain original morphological, physiological & genetic traits



Maintain genetic stability

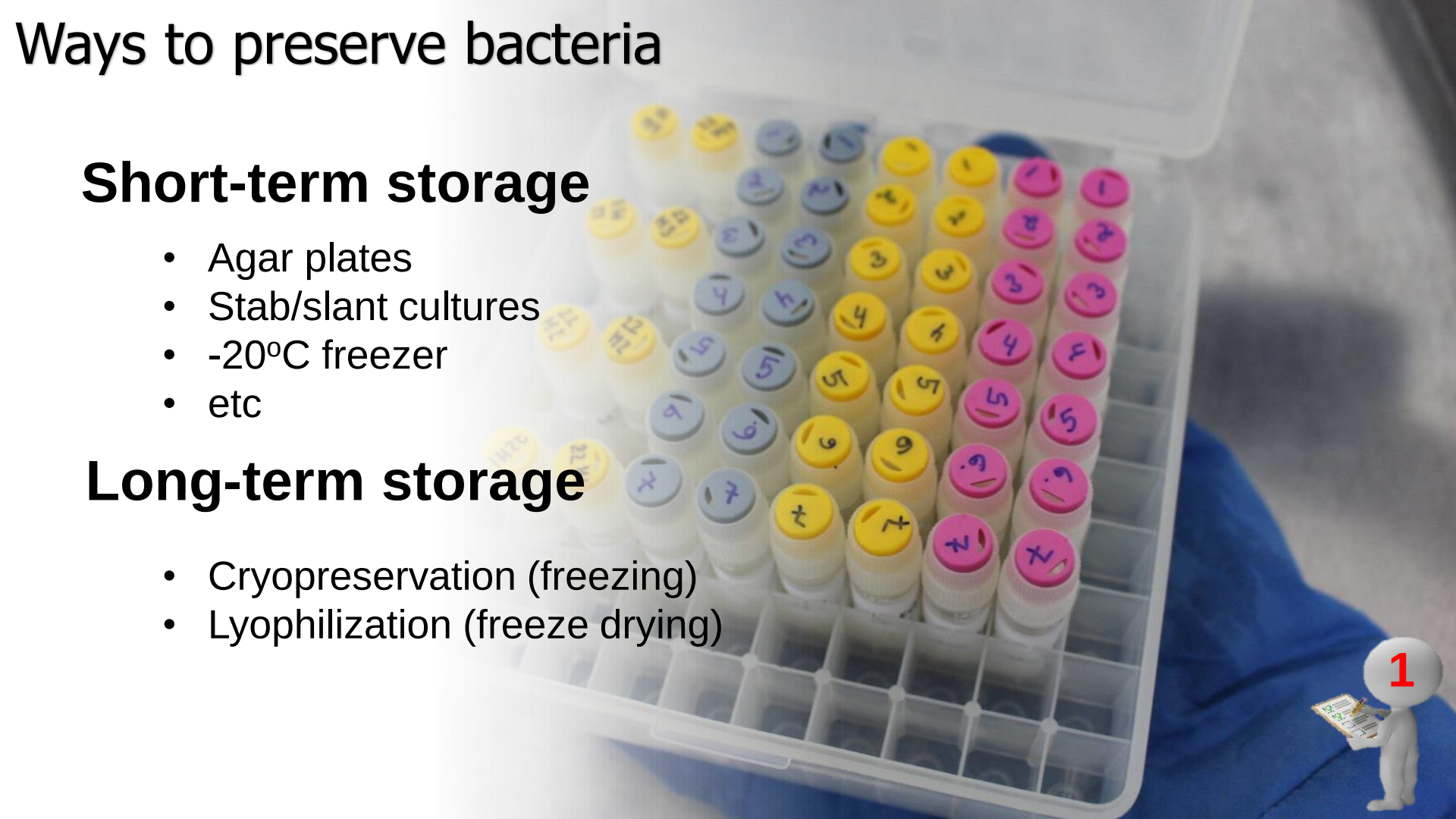
Ways to preserve bacteria

Short-term storage

- Agar plates
- Stab/slant cultures
- -20^{o}C freezer
- etc

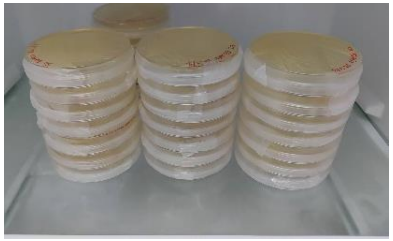
Long-term storage

- Cryopreservation (freezing)
- Lyophilization (freeze drying)



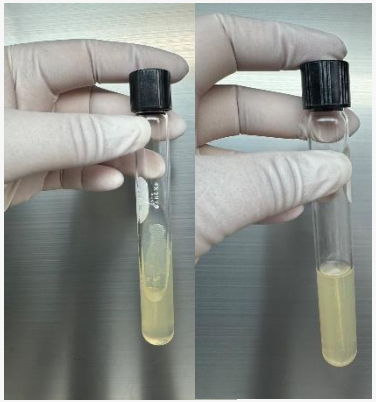
Short-term storage

Agar plate cultures



- viable for a few weeks when kept at 4°C.

Stab cultures



- Bacteria in the stab may live for at least 2 weeks when stored at 4 °C.
- **CLSI** - at 2°C to 8°C for up to four weeks.
- **ATCC** - at 2°C for up to a week

Freezing at -20°C and -40°C

- General purpose freezers
- viable for a few weeks to 1-2 years
- damages caused by ice crystal and electrolytes

Short-term storage

Quick and easy to recover bacterial cells

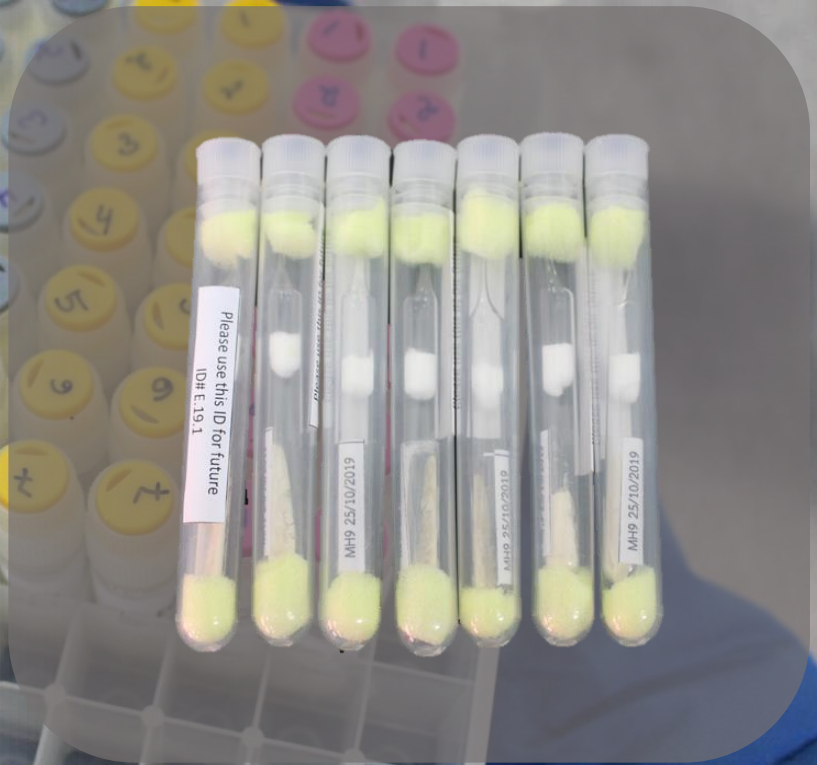
- Agar will dry over a prolonged period.
- The death of the stored bacteria due to nutrient starvation and toxic waste product build-up.
- Microorganisms may acquire unwanted genetic mutations.

- The stored bacterial isolates may exhibit genetic instabilities e.g., plasmid loss, point mutations, genome rearrangements etc.

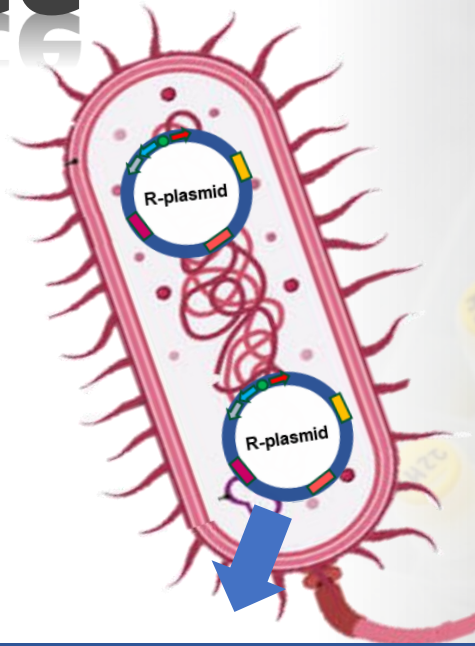
Long-term storage

- Ultra low temperature freezing
- Freeze-drying (Lyophilization)

- Less labor-intensive overtime
- Less laboratory space
- Less the chances of mutation events
- Higher genetic stability
- survival rates after freeze-drying vary with species.



Note



Bacteria carrying AMR determinants on plasmids may spontaneously loose the plasmid when stored at temperatures above −60°C and require a preservation method providing genetic stability.

Plasmid - An extrachromosomal DNA molecule independently replicate.

R plasmids - Plasmids that carry genes that provide resistance to various antibiotics

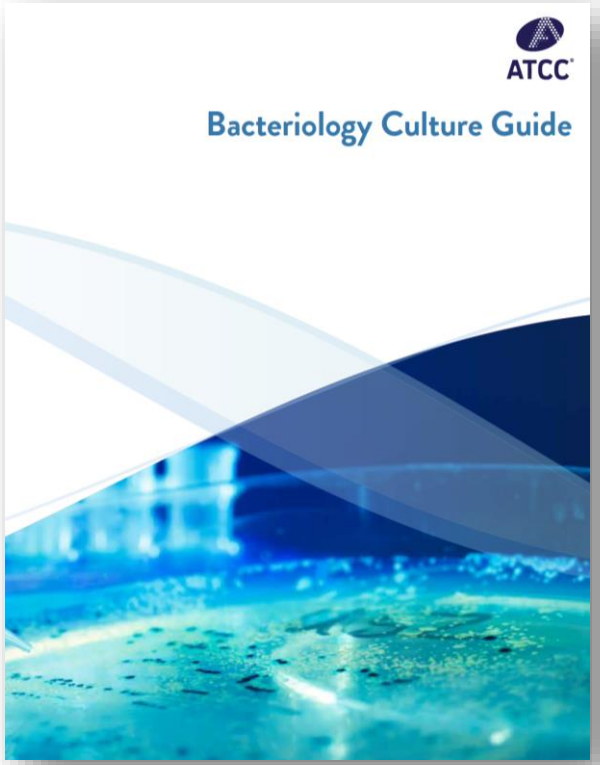
What storage methods to be used?

Think about the time scale of your experiments and plan ahead before preserving your bacteria.”

Methods		
Periodic subculture	Simple operation and convenient storage	Short-term storage and ease of mutation
Agar plate/stab	Simple operation and convenient storage	Short-term storage and ease of mutation
Freeze-drying	• Long-term storage • Wide range of strains	• High operation costs • Complicated operation
Cryopreservation	• Long-term storage • Wide application • Low-cost protocol	• Cryoprotectants required. • High energy consumption.

Guo et al 2020

Recommended storage methods



Methods commonly used:

- Cryopreservation (Freezing)
- Lyophilization (Freeze-drying)

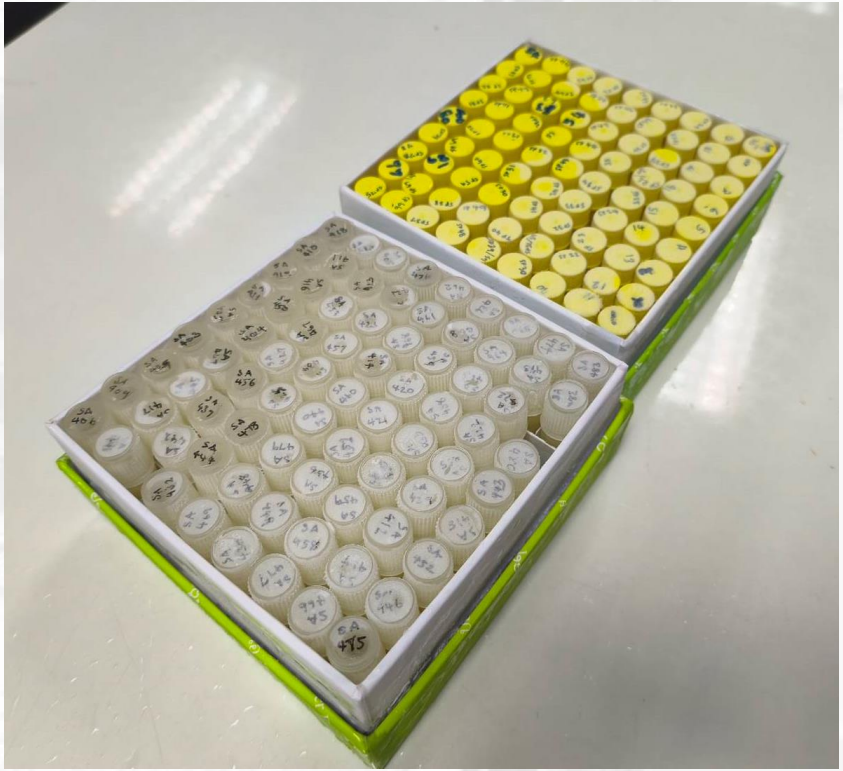
ATCC (American Type Culture Collection). 2015. ATCC® bacterial culture guide: tips and techniques for culturing bacteria and bacteriophages. Virginia, USA.

Cryopreservation (Freezing)

- Using ultralow temperature (cryogenic temperatures) generally at -80ºC and below to preserve biological materials

- -80°C is sufficient for storage of most bacteria for 5 years or less.

- -130°C is the critical temperature for long-term storage of biological materials.



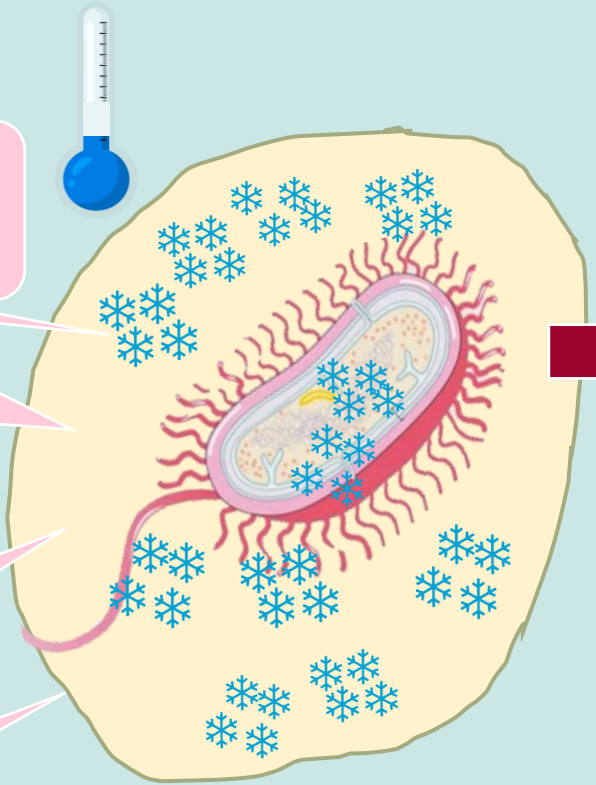
At below the freezing points,

1) Extracellular freezing trigger ice crystals formation outside of the cells. ❄

2) Solute concentration rises and extracellular osmolality increases

3) Hypertonic environment is created.

4) Water exits.



WITHOUT cryoprotectants

Osmotic stress

Cells shrink and cellular structures are damaged.

Mechanical stress

Ice crystal forms *inside* cells. Cellular membranes and other structures are damaged.

Decrease in viability

At below the freezing points,

- **To minimize the effects,**
  - Use a slow cooling rate, generally -1°C to -10°C per minute.
  - Add cryoprotectant agents

Standard procedure for cryopreservation

Cells in medium + a cryoprotectant in vials.

↓

Freeze cells slowly (at $-1°C\ min^{-1}$) until reach a temperature below -70°C

↓

Transfer vials to a liquid-nitrogen freezer to maintain cultures at temperatures below -130°C (-196 °C).

Freezing medium (Cryoprotectants)

Glycerol

- The optimal concentration of long-term glycerol storage is unknown.
- Most labs store bacteria in 15-25% glycerol with 50-75% stock.
- **ATCC** - a final concentration of 10% (20% stock).
- **CLSI** - a final concentration of 10%.

Other cryoprotectants

- 5-10% dimethyl sulfoxide (DMSO) (50% stock)
- 10% skim milk
- 50% fetal calf serum
- etc

At below the freezing points,

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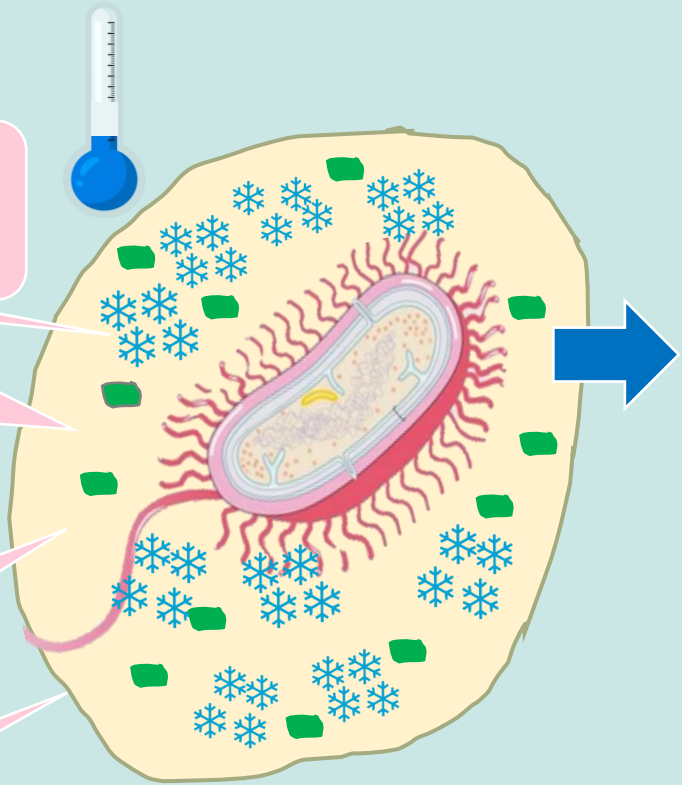
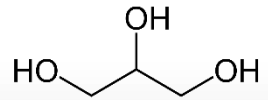


WITH cryoprotectants

Glycerol



Glycerol

- binds electrolytes to dilute their concentrations in the unfrozen fluid surrounding cells.
- binds water to delay ice crystal formation and slow freezing rate.

How to create glycerol stock

Grow bacteria on plate overnight (ON)

Inoculate 5 ml Luria-Bertani broth with a single colony

Option 1.

500 µl fresh ON culture

500 µl sterile 50% glycerol

Option 2.

800 µl fresh ON culture

200 µl sterile 75% glycerol

mix thoroughly

Store at -80°C.

https://www.addgene.org/protocols/create-glycerol-stock/

Freezing medium (Cryoprotectants)

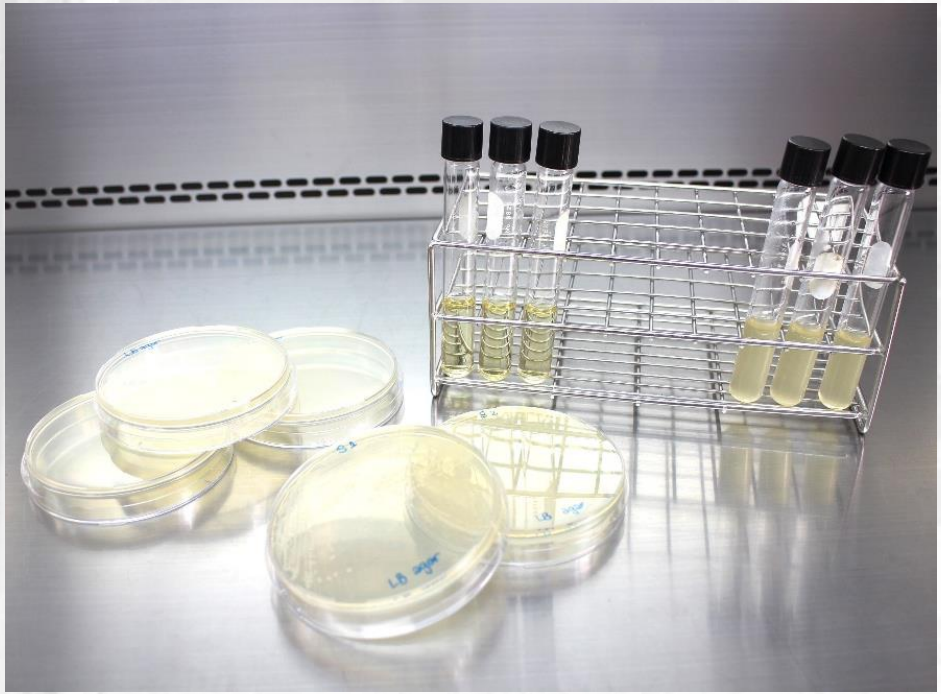
DMSO

- 5-10% dimethyl sulfoxide (DMSO) (50% stock)
- Alter cell permeability to facilitate movement of water out of the cells
- Use for glycerol sensitive cells

Skim milk

- 10-20% skim milk (50% stock)
- May affect the fatty acid content of cell membranes and help stabilize cell enzymes
- Commonly used for freeze drying

What culture media should be used?



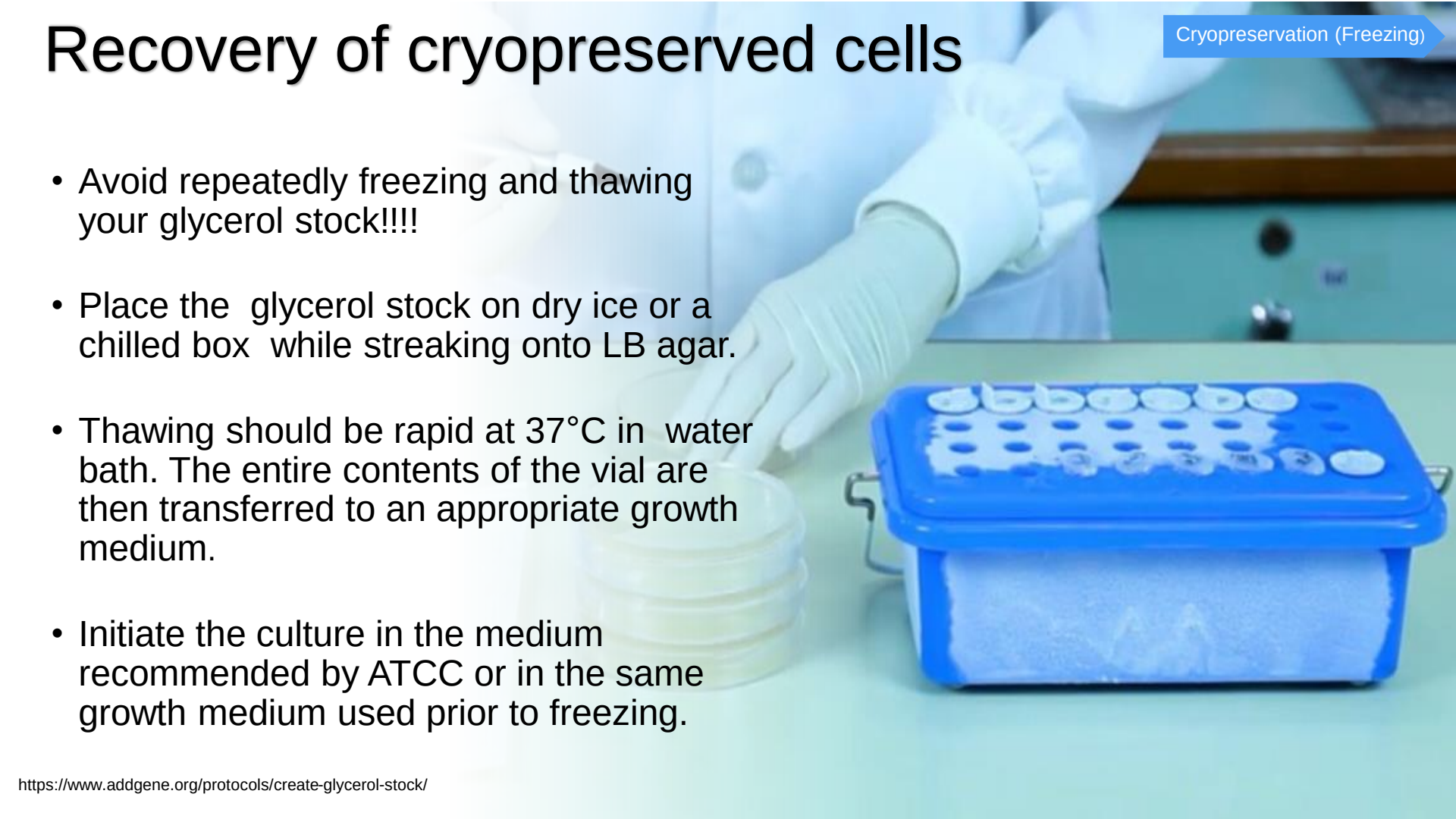
- Initiate the culture in the medium recommended by ATCC or in the same growth medium used prior to freezing.
- Incubate at optimum temperature & atmosphere
- Culture medium:
  - Luria Bertani broth
  - Tryptic soy broth
  - Others with equivalent nutrients
  - Additional supplements if required

Note

- Glycerol can be sterilized via autoclavation whereas DMSO must be sterilized by filtration.
- Use plugged or filtered tips if possible
- Make sure that you mix well and see one uniform solution.
- DO NOT store frozen cultures in a freezer with a defrost cycle. This will expose the cultures to higher temperatures.
- Avoid using snap cap tubes as they pop open accidentally.

Recovery of cryopreserved cells

- Avoid repeatedly freezing and thawing your glycerol stock!!!!
- Place the glycerol stock on dry ice or a chilled box while streaking onto LB agar.
- Thawing should be rapid at 37°C in water bath. The entire contents of the vial are then transferred to an appropriate growth medium.
- Initiate the culture in the medium recommended by ATCC or in the same growth medium used prior to freezing.



https://www.addgene.org/protocols/create-glycerol-stock/

Lyophilization (Freeze drying)

- Stable, dehydrated material with a relatively indefinite shelf-life when stored appropriately (2-8°C)
- Cryoprotectants vary based on the organism and method used - the most common one is 20% skim milk.
- Lower temperatures (-20°C or lower) for a longer shelf life.
- Keep sealed, away from moisture and oxygen and moisture

How to prepare lyophilized cells

Three steps:

pre-freezing to form a frozen structure

↓

primary drying to remove most water

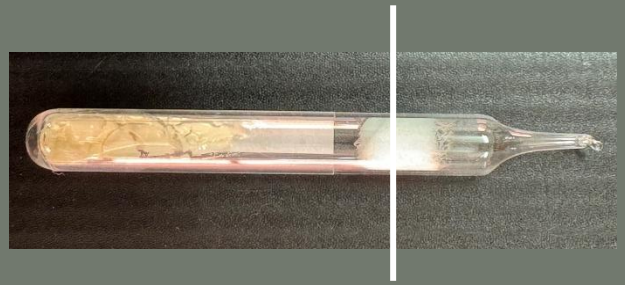
↓

secondary drying to remove bound water.

Lyophilized cells for EQAsia shipment

1)

2)



The ampoule should be cut in **the middle or below** the cotton wool plug.

How long can lyophilized cells be stored?

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2023, VOL. 37, NO. 1, 309–316
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Lyophilized *Escherichia coli* strains stored for 40–50 years: morphological, serotypic, biochemical characteristics and drug sensitivity

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ABSTRACT

Bacterial cultures are commonly preserved for long periods of time *via* freeze-drying (lyophilization). Lyophilized bacteria typically retain viability from 5 to 35 years. We investigated the vitality and preservation of some of the characteristic morphological, serotypic and biochemical features of 14 *Escherichia coli* strains following lyophilized storage for over 40–50 years. We also investigated their susceptibility to conventional antibiotics used in the therapy of infections caused by *Enterobacteriaceae* representatives. In our study, 14 strains of *E. coli* related to 11 serological types – O1, O2 (two strains), O5, O7, O11, O20, O25, O26, O29, O111 (two strains) and O125 (two strains) – were used. The lyophilized microorganism ampules were produced in the period of 1971 to 1973 and were stored at 4 °C in a microbial collection for educational purposes at the Medical College – Varna, Bulgaria. Control strains were *E. coli* ATCC25922, an *E. coli* strain (used for educational purposes) and three clinical *E. coli* isolates from urine and wound secretions. The *E. coli* strains stored for 40–50 years had preserved the studied morphological and biochemical characteristics, as well as those related to their antigenic characteristics and antibiotic sensitivity. Their susceptibility to the tested antimicrobials was analogical to the control reference strain *E. coli* ATCC25922, indicating that despite the long storage time, all strains retained and demonstrated the typical morphological, stereotypic and biochemical characteristics of the species.

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KEYWORDS
Culture storage; lyophilization; survival of microorganisms; *E. coli*; drug sensitivity

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Original Russian Text © M.B. Kupletskaya, A.I. Netrusov, 2011, published in Mikrobiologiya, 2011, Vol. 80, No. 6, pp. 842–846.

EXPERIMENTAL ARTICLES

Viability of Lyophilized Microorganisms after 50-Year Storage

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Received March 11, 2010

Abstract—The viability of lyophilized microorganisms belonging to different physiological groups was determined after 50-year storage at 2–4°C. During this period, the number of viable cells gradually decreased, in some cases, by 2–3 orders of magnitude. However, after 50-year storage, the ampoules contained considerable amounts of viable cells (in many cultures, 10^6–10^9 cells) that were quite sufficient for the culture maintenance. All the studied lyophilized microorganisms (pro- and eukaryotes) retained their viability after 50-year storage, a longer period than those known in literature.

Keywords: culture storage, lyophilization, survival of microorganisms.

DOI: 10.1134/S0026261711060129

Lyophilization is a widely-applied and effective method for the preservation of microorganisms. Lyophilization makes it possible to maintain microorganisms for a long time without transfers, which can change the properties of microorganisms [1, 2]. Lyophilization is employed in the studies of pathogenic bacteria as well as for the storage of microbial collections and for the preservation of active strains used in the production of vaccines and antibiotics. Industrial application of lyophilized lactic acid bacteria considerably reduces microbial contamination of dairy foods, thus increasing the quality and the shelf-life of the products [3, 4].

Lyophilization of bacteria is usually carried out with the use of protective media containing sugars and protein compounds [1, 5, 6], since the cells suspended

Protective media used in the DSMZ for the lyophilization of yeasts contained horse serum supplemented with 7.5% glucose or the mixture of 10% skimmed milk, 10% trehalose, and 10% sodium glutamate. Yeasts survive lyophilization poorly; only 1–30% of cells remained viable [9, 10]. However, the remaining cell amount is sufficient for the culture maintenance.

Yeasts are more tolerant to storage in liquid nitrogen than to lyophilization [9, 10]. However, the method of yeast storage in liquid nitrogen has a number of disadvantages, such as a risk of accidental defrosting and the impossibility of mailing the cultures; therefore, lyophilization is considered a more reliable method. In the DSMZ, lyophilized yeasts retained viability for 30 years without considerable

Approximate time bacterial culture remain viable under different storage condition

Storage	Temp (°C)	Approx. time
Agar plates	4	Few weeks
Agar stab/slant	4	1-4 weeks
General purpose freezer	-20/-40	Few weeks – 1 year
Freezer	-80	5 years or less
Super-cooled freezer	<-130 (-196)	Indefinitely
Freeze died	4	> 15 years

Preservation of reference strains

Strain definition:

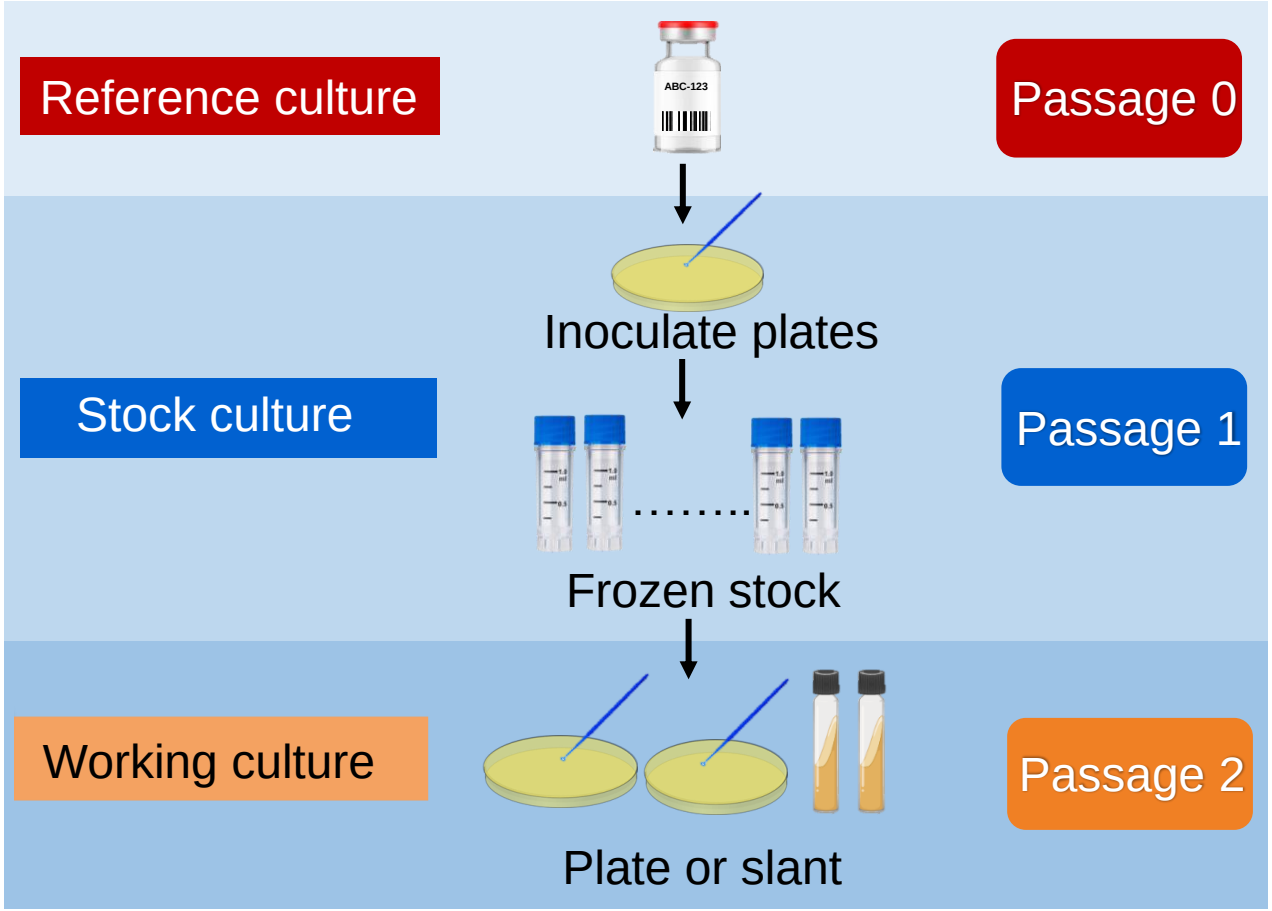
- Reference strains
- Reference culture
- Control strains
- Standard cultures
- Quality control (QC) strains

- Reference strains should come from a reliable source.
- **Both the CLSI and USP cite ATCC.**

QC Strain Culture Collections:

- American Type Culture Collection (ATCC) in the USA
- National Collection of Type Cultures (NCTC) in the UK
- Culture Collection of Switzerland (CCOS) Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in Germany
- Center of industrial Culture Collection (CICC) in China
- Thailand Institute of Scientific and Technological Research (TISTR) Culture Collection in Thailand

Seed lot system



Passage 1

ATCC reference strain is subcultured to several replicates at one time – all within one passage.

Stock cultures can be subcultured for reference cultures (monthly) and working cultures (weekly).

Passage 2

The replicates of the stock culture is subcultured to make replicates of working cultures.

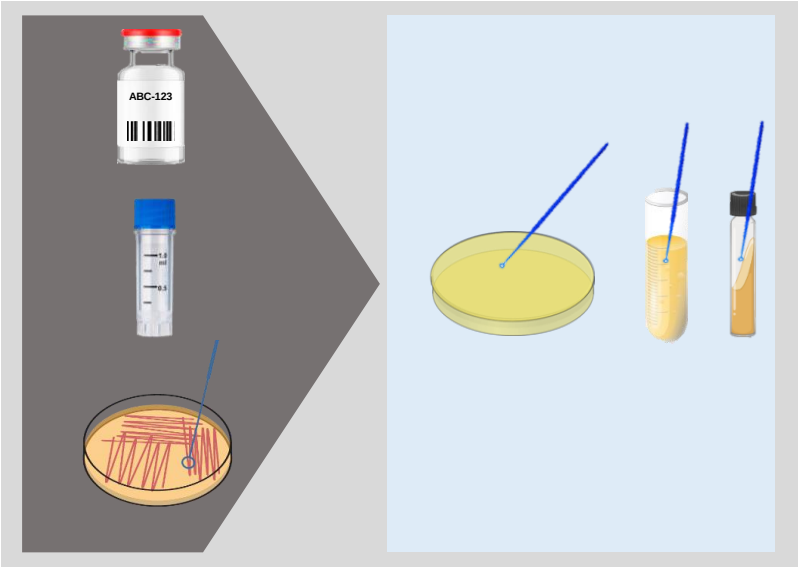
What is a passage?

USP 36-NF 31 <51> Antimicrobial Effectiveness Testing

"For the purposes of the test, one passage is defined as the transfer of organisms from an **established culture** to fresh medium".

USP <1117> Microbiological Best Laboratory Practices

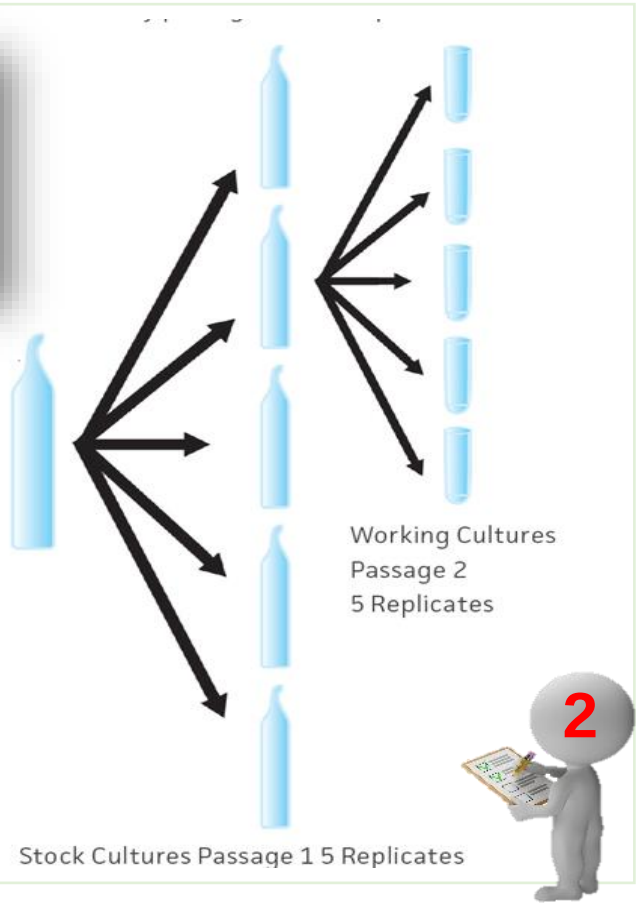
"One passage is defined as the transfer of organisms from a **viable culture** to fresh medium with growth of the microorganisms. Any form of subculturing is considered to be a transfer/passage"



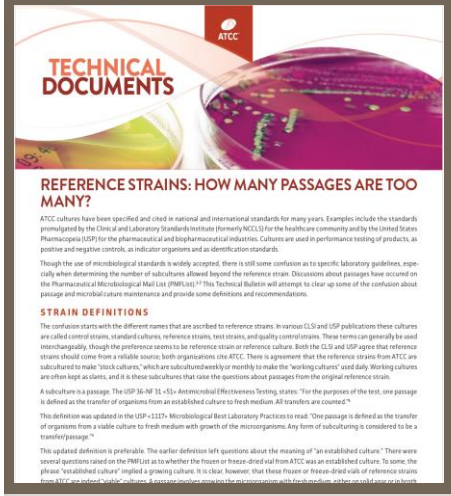
U.S. Pharmacopeia. Microbiological Best Laboratory Practices, <1117>. USP 36-NF 31, 2013.

How many passages are acceptable?

- **Passage numbers should be kept to a minimum.**
- Overpassaging will increase the chance of phenotypic variations, genetic drift, mutation and risk of contamination.
- It is important to safeguard the purity and identity of cultures and sub-cultures.



How many passages are acceptable?



- Most protocols accept to transfer the culture **5 passages (transfers)** from the original ATCC vial (Reference culture)

USP <1117 : "The working cultures used for testing **should not be more than 5 passages from the ATCC reference culture.**

ATCC : "…..with **no more than 5 passages from the ATCC reference culture** of USP standard.

Over the years, the CLSI recommendations have varied.!!!

Subcultured monthly, with no maximum number of passages noted.

Or

up to 3 subcultures of the stock cultures and up to 3 subcultures of the working cultures - add up to 7 passages from the original ATCC reference culture. .

Storage of frozen culture

CLSI

- Storage at −50°C to −70°C for one year or below −70°C indefinitely
- Slants is stored at 2°C to 8°C for up to four weeks
- Check CLSI for update

USP

- storage in liquid nitrogen or a mechanical freezer below −50°C.

ATCC

- Storage at the vapor phase of liquid nitrogen or a mechanical freezer at −80°C.
- Store freeze-dried cultures at 2°C to 8°C.
- 50% glycerol frozen stock at −20°C for less than one month.
- Slants may be stored at 2°C to 8°C for up to a week.

ATCC technical document, 2021

Note

Some QC strains, such as those with plasmid-mediated AMR, have been shown to spontaneously loose the plasmid when stored at temperatures above −60°C or if subcultured repeatedly.

Do not store frozen cultures in a freezer with a defrost cycle; this will expose the cultures to higher temperatures.

USP <1117> - Once opened, DO NOT refreeze unused suspension.

If a change in the organisms inherent susceptibility occurs, obtain a fresh stock culture or a new strain from a reference culture collection.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CHULALONGKORN UNIVERSITY
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http://www.cuvetamr.vet.chula.ac.th

PRESERVATION OF *CAMPYLOBACTER*

- **Preparation of storage medium (Skim milk with glycerol)**

Prepare 10 – 20% skim milk (70 ml) and glycerol (30 ml) in a separate bottle and autoclave at 121 °C for 15 minutes

Mix skim milk and glycerol thoroughly to make a culture storage medium

Distribute 1 ml of the storage medium containing skim milk and 30% glycerol (v/v) into a cryovial tube

PRESERVATION OF *CAMPYLOBACTER*

- **Preparation of bacterial stock culture**

STEP 1 Subculture a single colony of *Campylobacter* onto a blood agar plate and then incubate the plate at 42 °C for 24 hr under microaerobic conditions

STEP 2 Harvest the fresh overnight culture of *Campylobacter* on the blood agar plate (the entire lawn of bacterial growth)

STEP 3 Transfer the collected *Campylobacter* culture to a cryovial tube containing skim milk and 30% glycerol (v/v), mix thoroughly, and then freeze the stock culture at -80 °C

TIPS FOR *CAMPYLOBACTER* STOCK CULTURE PRESERVATION

- Good growth of *Campylobacter* before preservation (At least 1 – 2 loopful of culture per cryovial tube)
- Mix the culture and storage medium well before freezing
- Always keep the stock culture at -80 °C
- Avoid repeatedly freezing and thawing the stock culture
- Place the stock culture on ice during the re-subculturing to prevent the stock from completely defrosting