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Standard Practice for Preservation by Freezing, Freeze-Drying, and Low Temperature Maintenance of Bacteria, Fungi, Protista, Viruses, Genetic Elements, and Animal and Plant Tissues[1]

This standard is issued under the fixed designation E 1342; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

INTRODUCTION

Methods used for low temperature preservation of living biological systems include both freezing and freeze-drying. While in many cases other methods of preservation can be used, these low temperature methods provide the only real assurance of genetic stability. However, there are reports of damage to DNA as a result of freeze-drying **(1).**[2]

This practice assumes a basic knowledge of freezing and freeze-drying methods, and does not include specific methods used for freezing and freeze-drying.

1. Scope

1.1 This practice covers the handling of microorganisms (bacteria, fungi, and protista), viruses, genetic elements (nucleic acids and plasmids), and animal and plant cell tissues (cell lines), during and after freezing and storage at cryogenic temperatures.

1.2 This practice also covers the handling of microorganisms, viruses, and genetic elements in the host cell during and after freeze-drying.

1.3 While this practice does not cover the specific methodology used to freeze and freeze-dry microorganisms and cell lines, the safety aspects of handling microorganisms during freezing and freeze-drying procedures, and during storage at cryogenic temperatures, are covered. Other guidelines must also be adhered to regarding the handling of hazardous materials **(2)**.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* For specific hazard statements see Section 6.

2. Referenced Documents

2.1 *ASTM Standards:*

E 1564 Guide for Design and Maintenance of Low-Temperature Storage Facilities for Maintaining Cryopreserved Biological Materials[3]

E 1565 Guide for Inventory Control and Handling of Biological Materials Maintained at Low Temperatures[3]

E 1566 Guide for Handling Hazardous Biological Materials in Liquid Nitrogen[3]

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *cryogenic temperatures*—temperatures below or equal to −100°C.

3.1.2 *cryoprotectant*—a chemical substance used to protect cells during freezing and rewarming.

3.1.3 *eutectic temperature*—the temperature below which all liquid portions of an aqueous suspension have entered the solid phase.

3.1.4 *freeze-drying*—sublimation of water from a frozen aqueous suspension.

3.1.5 *freezing*—lowering the temperature of an aqueous suspension to a point at or below the temperature of ice crystal formation.

3.1.6 *low temperature preservation*—stabilizing viable or biologically active material by freezing or freeze-drying.

3.1.7 *vitrification*—solidification of an aqueous suspension at low temperatures without the formation of ice crystals.

4. Significance and Use

4.1 The staiblity of cell populations and genetic elements at low temperatures can be affected by the methods used to

[1] This practice is under the jurisdiction of ASTM Committee E48 on Biotechnology and is the direct responsibility of Subcommittee E48.02 on Characterization and Identification of Biological Systems.

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[2] The boldface numbers in parentheses refer to the list of references at the end of this practice.

[3] *Annual Book of ASTM Standards*, Vol 11.05.

preserve the material, and by procedures used in handling the material during storage **(3)**.

4.2 The intent of this practice is to outline procedures that can minimize the adverse effects of handling biological materials during low temperature preservation and maintenance.

5. Freezing

5.1 To successfully freeze living cells, a chemical agent (that is, a cryoprotectant) is often used to protect the cells during cooling and rewarming. The cells are harvested and the cryoprotectant is added just prior to freezing. Exposure to the cryoprotectant is limited to no more than 1 to 2 h before beginning cooling of the cells. The optimal exposure time varies depending on the material being preserved.

5.2 For most microorganisms and cell lines, the optimum cooling rate when using a single cryoprotectant is a uniform 1 to 10°C/min to at least −35°C. Cooling must continue to at least −35° at this rate before exposing cells to lower temperatures. Below −35°C more rapid cooling (that is, 50 to 100°C/min to cryogenic temperatures) is preferred. With aggregates of cells, a mixture of cryoprotectants that solidifies at low temperatures without the formation of ice (vitrification) may be more optimal **(4,5)**. When moving frozen material from one container to another, such as from a freezing unit to a liquid nitrogen freezer, make sure that exposure to ambient temperatures is no more than 5 to 10 s. If material must be moved more than a few ft, use a styrofoam carrier containing dry ice or liquid nitrogen to maintain the temperature of the frozen material during transport. Make sure that the container holding the frozen material is in direct contact with the dry ice or liquid nitrogen.

5.3 During storage at cryogenic temperatures the frozen material must be maintained below a critical predetermined temperature suitable for the material being stored. An increase in temperature above the critical temperature can result in ice crystal formation and a consequent loss of viability.

5.3.1 When using mechanical freezers, care must be taken not to warm the internal environment of the unit more than 10°C when adding or retrieving material. Minimize the interval during which the door to the unit is open. Make sure that removal and replacement of frozen material during retrieval operations is minimized. Design an inventory system providing ready access to all material in the unit to avoid inadvertent warming of preserved material during retrieval of other items.

5.3.2 When using all vapor storage liquid nitrogen freezers the gradient in the vapor can be disrupted during stocking and retrieval activities, and is more severe when the liquid level in the freezer is too low. Maintain the liquid in the unit at a level that ensures a temperature no warmer than −150°C in the highest portion of the inventory space during the longest period of operation with the lid off. Freezers should be properly validated to assure adequate operating temperatures **(6)**.

5.4 Minimize handling of frozen specimens during retrieval. To limit the working time in a low temperature freezer, design an inventory system that allows pinpointing of the exact location of a single ampoule or vial. During retrieval of an ampoule or vial, make sure that all material remains at a relatively constant temperature. Handling procedures require that boxes or other containers remain at the coldest temperature of the working area of the freezer during retrieval of a single vial. While this may not always be possible, take precautions to ensure that only the vial being retrieved is exposed to warmer temperatures as it is transferred to a portable container. When transferring material from a low temperature freezer to a portable container for transporting, all operations must be made quickly to minimize exposure of the retrieved vial to ambient temperatures.

5.5 Several factors can affect the recovery of cells during and after freezing. The type of cells, age of the cells, growth conditions, cell concentration, rate of cooling, type and concentration of the cryoprotectant and storage temperature, are all factors that must be considered when freezing living cells.

6. Freeze-Drying

6.1 During dispensing of microbial suspensions for freeze-drying, minimize exposure to ambient temperatures to limit changes in population size and production of extracellular products that may affect the freeze-drying process and recovery of the microorganisms. Dispensing should immediately follow harvesting, and when large volumes of material are dispensed, make sure that the cell suspensions are constantly stirred and maintained at 2 to 8°C during the dispensing operation to ensure uniformity of the product.

6.2 *Maintenance During Freeze-Drying*:

6.2.1 Optimum temperatures for freeze-drying are at or just below the eutectic temperature of the suspension to be freeze-dried. For most microbial suspensions this temperature is generally warmer than −40°C, a temperature suitable for ice crystal formation that is destructive to the viability of most microbes. Therefore, accomplish drying in as short a time as possible. To ensure adequate heat transfer, use freeze-drying trays with removable bottoms to allow direct vial contact with the shelf. The type of glass vial can also affect the heat transfer, as molded vials may not uniformly contact the shelf.

6.2.2 During the freeze-drying process a temperature differential of at least 20°C between the product and condenser is required to ensure an adequate vapor pressure of less than 10 µm Hg (0.01 torr). During freeze-drying maintain a pressure of 50 µm Hg (0.05 torr) or below. When elevated pressure drying can be used, it will accelerate the rate of drying **(7)**. However to ensure adequate drying the pressure must be reduced to below 50 µm Hg during the final stages of the drying process, and the shelf raised to ambient temperature.

6.2.3 Several closure systems can be used following freeze-drying including closing in a vacuum, under reduced pressure, or at atmospheric pressure. When closing under reduced pressure, or at atmospheric pressure, backfilling must be accomplished with an inert gas (that is, not O_2) under aseptic conditions. Backfill the containers following freeze-drying through a 0.22-µm filter assembly to ensure sterility of the inert gas, and through a liquid nitrogen cold trap to prevent warming of the condenser and backflushing of moisture to the product. The cold trap is not essential when using freeze-dryers with external condensers.

6.2.4 Microbial strains cannot be mixed in a freeze-drying operation unless bacterial filters are used to prevent cross contamination. When freeze-drying without a filtration system, cross contamination can occur **(8)** and contamination of the

freeze-drying system occurs. When filters are not used, the freeze-drying system must be decontaminated after each freeze-drying run.

6.3 During storage freeze-dried material must be maintained at a temperature that does not fluctuate more than ±10°C under conditions free of oxygen, light, and moisture. The lower the storage temperature, the longer the expected shelf life of the freeze-dried material. Freeze-dried material should be stored at 2 to 8°C or lower. Thoroughly dry rubber closures prior to use. Do not store rubber stoppered vials at liquid nitrogen temperatures **(9)**.

6.4 Several factors can affect the recovery of cells during and after freeze-drying. The type of cells, age of the cells, growth conditions, cell concentration, rate of cooling, type and concentration of suspending medium, freeze-drying conditions, storage temperature, container integrity, residual moisture, and the method of reconstitution, are all factors that must be considered when freeze-drying living cells.

7. Hazards

7.1 **Warning**—gloves shall be worn when working at liquid nitrogen temperatures (below −150°C) to protect hands and arms from the extremely cold temperatures. A face shield shall also be worn when handling material in the liquid phase of a liquid nitrogen freezer, and especially when working with torch-sealed glass ampoules. Improperly sealed glass ampoules can have microscopic openings that can allow liquid nitrogen to leak into the ampoules **(10)**. **Warning**—when these ampoules are retrieved to warmer temperatures an explosion can result, spewing glass shards and potentially hazardous infectious material. Certain types of plastic vials can also explode after storage in liquid nitrogen. Do not store hazardous biological agents in the liquid phase of a liquid nitrogen unit.

7.2 Always locate liquid nitrogen refrigerators in a well ventilated area, and always use low-pressure liquid nitrogen supply tanks to recharge the refrigerators. High-pressure tanks can be destructive to automatic-fill units, and can be potentially dangerous to personnel when used to fill manually.

7.3 Dry ice is often used to transport frozen specimens, and care must be taken to protect the skin from contact with dry ice. Do not use dry ice in an enclosed area. When frozen specimens are shipped in metal cans, care must be taken to ensure that no dry ice is present in the can before it is sealed. **Warning**—hermetically sealed metal cans containing even small amounts of dry ice can explode when they are opened.

7.4 When freeze-drying microorganisms in tray dryers without using bacterial filters in the vessels, the entire contents of the freeze-dryer, (including the vessels containing the product), are potentially contaminated at the completion of the freeze-drying run. Use gloves and respiratory protection in handling product removed from the freeze-dryer, and the outside of the product containers must be decontaminated prior to further handling.

7.5 Decontamination of the chamber of a tray dryer can be accomplished using ethylene oxide. In most systems this is the only method currently available. Ethylene oxide must be used in well-ventilated areas, and an adequate exhaust system must be available for removing residual ethylene oxide from the freeze-drying chamber. Periodic monitoring of personnel and equipment as a check on procedures is recommended. Make sure that residual ethylene oxide levels are as low as practicable to ensure compliance with OSHA regulations regarding personnel exposure.

8. Keywords

8.1 animal tissue; bacteria; biotechnology; cryopreserved; freeze-drying; freezing; fungi; genetic elements; microorganisms; protista

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