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3-D printing provides a novel approach for standardization and reproducibility of freezing devices

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ABSTRACT

Cryopreservation has become an important and accepted tool for long-term germplasm conservation of animals and plants. To protect genetic resources, repositories have been developed with national and international cooperation. For a repository to be effective, the genetic material submitted must be of good quality and comparable to other submissions. However, due to a variety of reasons, including constraints in knowledge and available resources, cryopreservation methods for aquatic species vary widely across user groups which reduces reproducibility and weakens quality control. Herein we describe a standardizable freezing device produced using 3-dimensional (3-D) printing and introduce the concept of network sharing to achieve aggregate high-throughput cryopreservation for aquatic species. The objectives were to: 1) adapt widely available polystyrene foam products that would be inexpensive, portable, and provide adequate work space; 2) develop a design suitable for 3-D printing that could provide multiple configurations, be inexpensive, and easy to use, and 3) evaluate various configurations to attain freezing rates suitable for various common cryopreservation containers. Through this approach, identical components can be accessed globally, and we demonstrated that 3-D printers can be used to fabricate parts for standardizable freezing devices yielding relevant and reproducible cooling rates across users. With standardized devices for freezing, methods and samples can harmonize into an aggregated high-throughput pathway not currently available for aquatic species repository development.

1. Introduction

Environmental changes, pollution, and overharvesting caused by human activities continue to threaten and reduce the diversity of natural fish and shellfish populations [13,27]. At the same time, medical research is generating thousands of new transgenic and mutant lines of model species such as Zebrafish [10,16]. Cryopreservation of germplasm is a powerful tool to preserve genetic resources such as these for conservation, stock enhancement, biomedical research, and aquaculture [13,25]. It has been applied around the world for numerous aquatic species [6,24]. However, if cryopreservation is going to be an effective tool, reproducibility must be improved [9], and protocols and equipment need to be standardized and harmonized across user groups.

Although there are published studies on more than 200 aquatic species demonstrating the success of cryopreservation [15,34], there is little uniformity among those reports, and the ability to repeat or compare research is low [31]. Among the wide variety of methods, there are published reports demonstrating the capability of freezing spermatozoa in nitrogen vapor using a portable polystyrene foam container [3,22,26], which enables application in the field and in small-scale production. The use of a polystyrene foam box also provides a low-cost alternative for cryopreservation compared to a programmable freezer. However, most of these studies fail to report important information such as cooling curves and the dimensions of the box, depth of liquid nitrogen, or height above the nitrogen at which the samples were frozen [11,14,17]. Because the vapor-phase temperature gradient inside a polystyrene foam box can be influenced by factors such as wall thickness, volume proportion of liquid nitrogen to air space, and sample distance

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above the liquid nitrogen [2,29], the lack of these measurements and cooling curves makes comparisons problematic and often disallows reproducibility among studies.

Indeed, many “standard protocols” in cryopreservation of aquatic species often prove to be non-transferrable due to lack of access to specific information, tools, or equipment [30,37]. With the advent of 3-dimensional (3-D) printing technology, variations among devices can be removed as a bottleneck that prevents the merging of cryopreserved products from different sources under a uniform standard. Thermoplastics such as polylactic acid (PLA) are well suited to use for cryogenic activities [33] and are routinely used in 3-D printers. In addition, the philosophy behind open-source 3-D printing could be used in forming a genetic resource community to share and improve cryopreservation experiences with the use of standardized or harmonized devices and protocols. As such, our goal was to develop an approach that could assist small-scale cryopreservation in achieving standardizable aggregate high-throughput. This aggregate pathway is an approach to support repository development by combining defined-quality samples from multiple locations into a unified repository system. The objectives were to: 1) adapt widely available polystyrene foam insulating containers that would be inexpensive, portable, and provide adequate work space; 2) develop a design suitable for 3-D printing that could provide multiple configurations, be inexpensive, and easy to use, and 3) evaluate various configurations to attain freezing rates suitable for various common cryopreservation containers. In this study, we introduce a 3-D printed freezing device to serve as a conceptual model to encourage development of similar devices providing a range of cooling rates based on commonly used containers for aquatic species cryopreservation, and introduce the prospect of using 3-D printing to enhance standardization and harmonization among members of the developing aquatic cryopreservation community.

2. Materials and methods

2.1. Insulating boxes

Products from a large commercial supplier (Polar Tech Industries, Genoa, IL) were selected to provide insulation during cryopreservation due to their wide availability and low cost. Sufficient insulation was achieved by inserting the Insulated Bio Foam Container (Item # 214F) with 31.8-mm wall thickness into a Nestable Insulated Shipper (Item # NS-9KD) with 25.4-mm wall thickness. The combination provided a 57.2-mm total wall thickness and a 12.7-mm layer of air between the two boxes. An outer cardboard carton was used to protect the boxes from punctures and to facilitate shipping. The Bio Foam container had a height of 178 mm and held ~5 L of liquid nitrogen when filled half-way. The liquid nitrogen level inside dropped at a rate of ~10 mm per hr when held at room temperature (23 °C) with both boxes covered with the provided lids.

The temperature profile within the insulating boxes was recorded using 4 type-T thermocouples (Omega Engineering, Stamford, CT) and a multichannel temperature data logger (UEi DT304, UEi Test Instruments, Beaverton, OR). The inner Bio Foam container was filled with liquid nitrogen to a depth of 102 mm. Thermocouples were positioned at 19 mm, 38 mm, 57 mm, and 76 mm above the liquid nitrogen surface and temperatures were recorded for 30 min with the polystyrene lids in place.

2.2. Device design

The major components of the freezing device were two horizontal platforms, two sets of connecting columns of different heights (40 mm and 50 mm), an extruded (closed-cell) polystyrene foam raft, a lifter, and the two nested expanded polystyrene (e.g., Styrofoam) foam boxes described above (Fig. 1). All freezing device components were designed using the free version software Sketchup 8 (Trimble Navigation, Sunnyvale, CA, version 8.0.16846) and were fabricated by 3-D printing using polylactic acid (PLA) filament (Makerbot Industries, Brooklyn, NY) with a fused deposition modeling (FDM) MakerBot Replicator 2 (Makerbot Industries, Brooklyn, NY) 3-D printer. This PLA filament was chosen over acrylonitrile butadiene styrene (ABS) filament because of our observations of its better properties at cryogenic temperatures (−196 °C) [33]. The polystyrene raft was cut from a 12.7-mm thick (R-3) faced extruded polystyrene foam insulation board (STYROFOAM™ Brand DURAMATE™ Plus, Dow Chemical Company, Midland, MI) available at home improvement stores. A 127-mm x 127-mm centered square was cut in the raft that could be removed or replaced as needed (Fig. 1). Removal of the inner raft square decreased the total surface area of the polystyrene raft from 542 cm^2 to 155 cm^2. This allowed for more interaction of nitrogen vapor with the containers increasing the cooling rate for use with French straws. Cryovials and Bank-it tubes only used the full sized 542 cm^2 raft. This design provided a standardized means to suspend samples over liquid nitrogen to provide reproducible cooling rates that could be adjusted easily by changing the device configurations.

For use, liquid nitrogen was added to the inner Bio Foam container at a depth of about 100 mm (95–105 mm, about 5 L). The depth of liquid nitrogen was determined by slowly inserting a black ruler to the bottom, removing it, and viewing the frozen condensate. In general, a lowering of the liquid nitrogen level by 5–10 mm per freezing run did not affect performance. After each run, the liquid nitrogen level was readjusted. The raft lifter and raft were lowered into the liquid nitrogen for pre-cooling and both polystyrene boxes were covered and allowed to equilibrate for a minimum of 5 min. This stabilized nitrogen vapor levels inside the freezing chamber and cooled the outer container layer. If the lifter and raft were not pre-cooled, the liquid nitrogen would boil when lowered with samples and cause improper cooling. After equilibration, all polystyrene boxes remained covered until freezing began. The sample containers were placed on the freezing platform and held at 4 °C. For our trials, the freezing platform was fully loaded with the specific container types being tested. To begin freezing, the lifter with raft were carefully raised from the liquid nitrogen, the freezing platform was placed on top of the raft and the entire apparatus was lowered onto the surface of the liquid nitrogen. The lifter sank into the liquid nitrogen, while the freezing platform remained on the surface, providing a reproducible cooling configuration for all 6 combinations of platform and raft options using four different container types (Table 1). The two boxes were covered until the full freezing profile was achieved (according to cooling rate, usually no more than 20 min). After freezing, the samples were plunged into liquid nitrogen while containers were sorted for storage in dewars.

2.3. Performance testing

Comprehensive tests were performed using 4 type-T thermocouples and a multichannel temperature data logger to record the temperature profiles (measured to 0.1 °C) of the different combinations of variables. The tests were performed indoors at room temperature of 22–25 °C. Thermocouples were inserted into containers filled with Hanks' balanced salt solution, a commonly used extender solution for cryopreservation of fish sperm [35], at an osmolality of 300 mOsmol/kg (HBSS300: 0.137 M NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 1.0 mM MgSO4, 0.25 mM Na2HPO4, 0.44 mM KH2PO4, 4.2 mM NaHCO3, and 5.55 mM glucose, pH 7.2) [32] and