



Cryopreservation of Potato Shoot Tips for Long-Term Storage

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Abstract

Cryopreservation is currently the only method which allows long-term conservation of living clonal plant material in the vapor or liquid phase of nitrogen (at -140 to -196 °C) allowing tissue to be viable for decades or perhaps centuries. Specifically, for species with recalcitrant seeds or requiring constant vegetative propagation, it is the method of choice for the long-term conservation of its genetic resources. The protocol described here is a modification of a previously developed plant vitrification solution 2 (PVS2)—droplet vitrification method of potato shoot tips, adapted from *Musa* species. Utilizing this protocol, the International Potato Center (CIP) has successfully stored in the cryobank more than 3000 cultivated potato accessions, belonging to seven species and nine different taxa [16], originating principally from ten countries in South and Central America. As part of CIP's quality management system, all vegetative material placed in cryo is routinely subsampled, thawed, and assessed to confirm that whole plantlets can be produced after storage in liquid nitrogen. Complete plant recovery rates of thawed shoot tips range from 20% to 100% (average rate: 60%). This chapter describes the complete set of steps from the routine procedure of cryopreserving potato shoot tips for long-term conservation.

Key words Cryopreservation, Potato (*Solanum species*), PVS2, Droplet vitrification, Cryobank

1 Introduction

The secure and efficient conservation of genetic resources of important staple crops such as potato, before they are lost in the natural environment, is essential to ensure the welfare of future generations, especially in times of climate change and lack of food security in many parts of the developing world. Conservation of genetic resources allows the development of new varieties that can resist environmental challenges and provide better nutrition for

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humanity, but this can only occur through preservation of diversity and subsequent efforts in breeding and selection. Cryopreservation offers the best available option for the secure, space-efficient, and low-cost conservation of clonally propagated species for the long term (decades to centuries), without the need of subsequent subculturing or renewal of the plants. Although the introduction of a new accession to the cryobank is expensive (\$400–500 USD/accession), maintaining it in the cryobank is not expensive (\$2 USD/accession/year), compared to a yearly cost of \$50–60 USD for conserving an accession in the *in vitro* bank. After a conservation period of ~8–10 years, cryobank storage becomes cheaper than *in vitro* banking.

Different cryopreservation methods have been developed for many plant species using desiccation, slow cooling, vitrification, encapsulation-dehydration, encapsulation-vitrification, droplet vitrification, and V- and D-plate [1–3]. All of these protocols strive to avoid the formation of large intracellular ice crystals during freezing and thawing as it is harmful to the cell membranes and generally leads to cell death (apoptosis). Ice crystallization can only be avoided when a glassy state is reached on the intracellular level at freezing, mainly by increasing the viscosity to a point that the system behaves like it is in a solid state (called glassy state or vitrification) [4]. Plant tissue is generally treated with a mix of cryoprotectants, e.g., glycerol, dimethyl sulfoxide (DMSO), and ethylene glycol, to achieve vitrification. The most famous and frequently used cryoprotectant solution for plant genetic resources is called plant vitrification solution 2 (PVS2). It was developed nearly three decades ago for nucellar cells of navel orange by a Japanese research group [5]. A few years later, a German group modified the droplet vitrification method, originally developed for cassava, as an ultrarapid cooling method for potato [6–8]. The droplet method uses a small strip of aluminum foil with a droplet of cryoprotectant. The samples (generally, shoot tips of a size of 0.8–2.0 mm) are placed into a droplet of cryoprotectant and then quickly plunged into liquid nitrogen. Initially DMSO was used as a cryoprotectant, but later it was broadly replaced by PVS2 and its application led to a breakthrough of successful cryopreservation of many crops [2, 9, 10].

The International Potato Center (CIP) adapted the PVS2-droplet vitrification method in 2004 for potato and has continuously improved its efficacy and efficiency over the last 15 years [1, 11–15]. In general, the main steps in this protocol include *in vitro* propagation, pre-culture, shoot-tip excision, cryoprotectant treatment of shoot tips, freezing, thawing, and plant recovery assessment in order to determine the success of each cryo-run (Fig. 1). One single person can cryopreserve 300–450 potato shoot tips during an 8-h working day (including shoot-tip excision, cryoprotection, freezing in liquid nitrogen, and transitory storage). CIP's cryopreservation protocol is applicable to a wide range of potato species and genotypes (~3250) [16] and shows a high

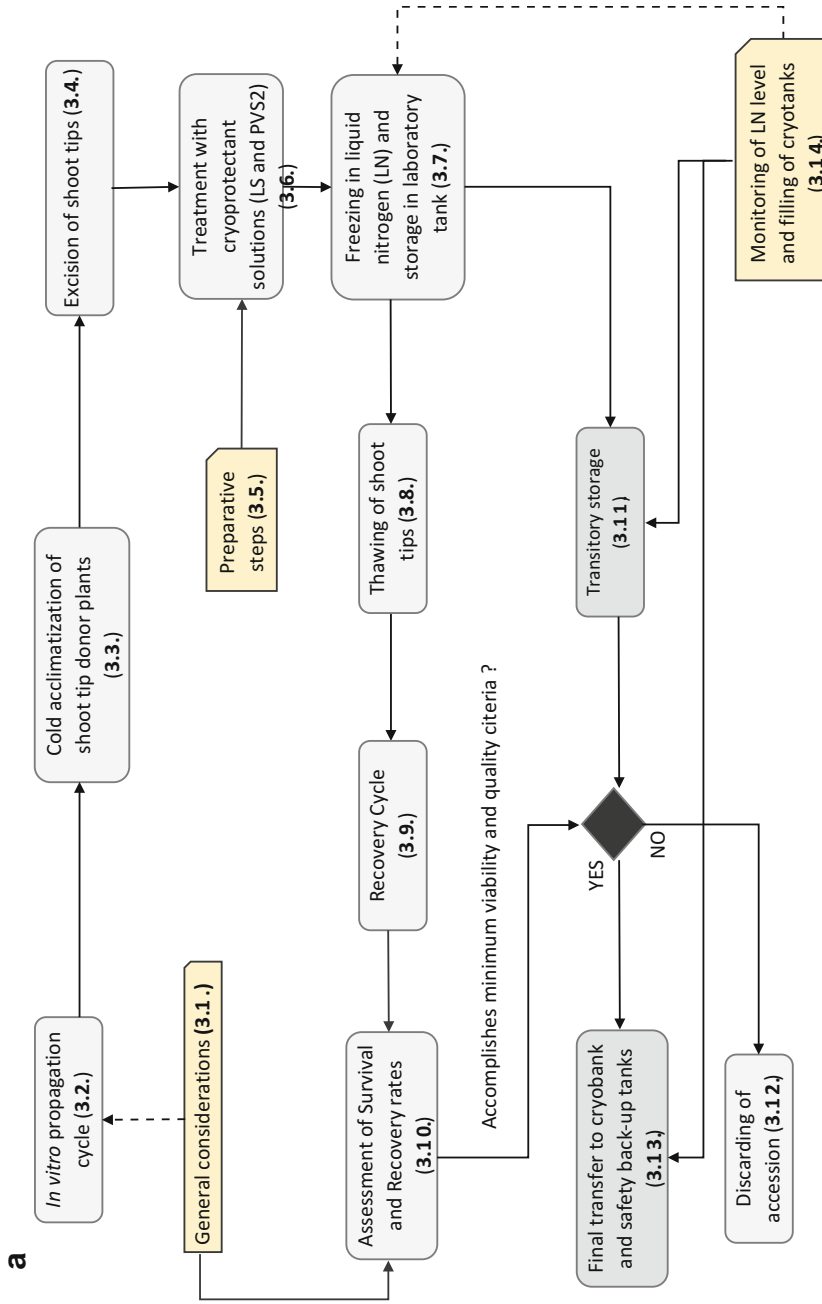


Fig. 1 (a) Simplified workflow of the potato cryopreservation process applied at the International Potato Center (CIP). Corresponding method sections of each step are specified in parentheses. Principal steps are connected with a continuous arrow line and peripheral steps with dotted arrow lines. **(b)** Simplified scheme of the potato cryopreservation process applied at the International Potato Center (CIP). Corresponding method sections of each step are specified in parentheses. Transfer events between tanks are presented with dotted arrow lines

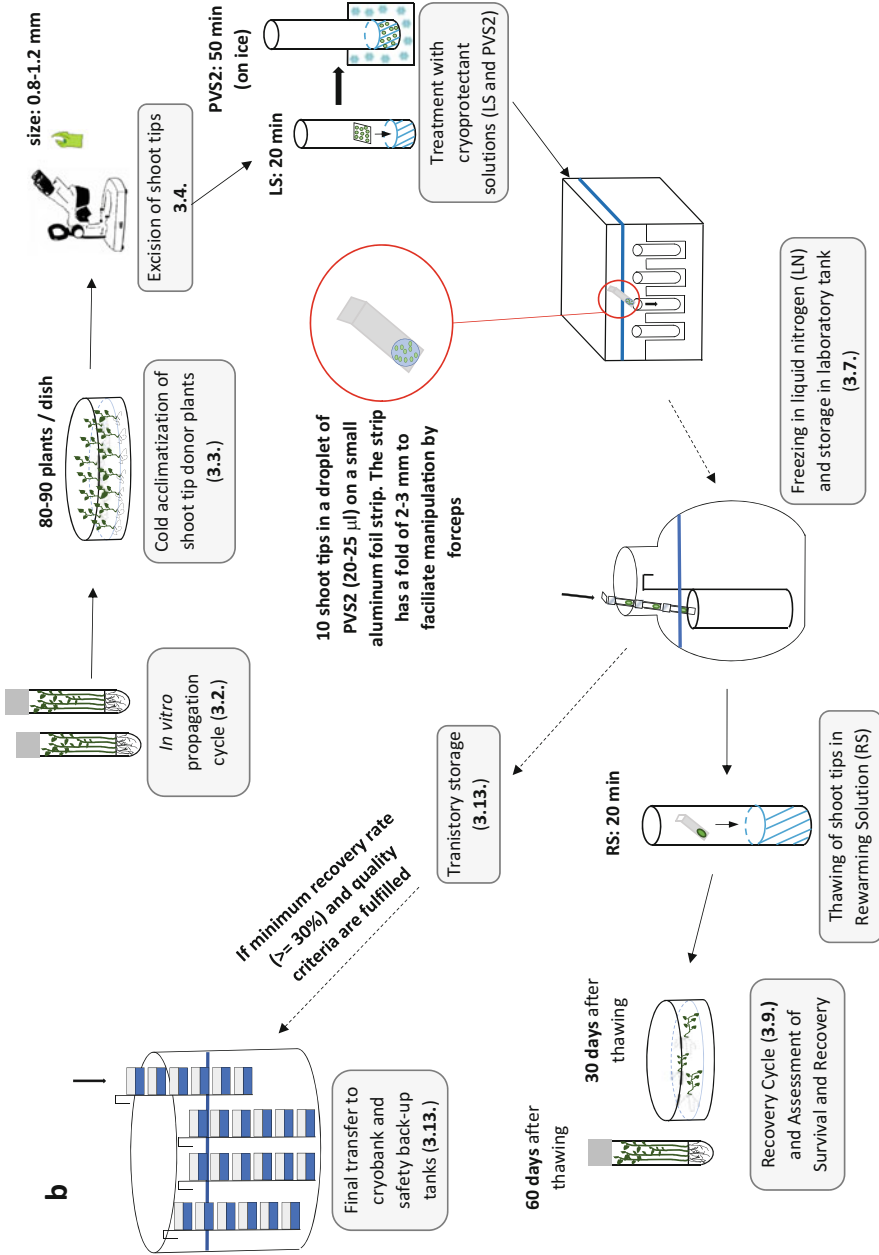


Fig. 1 (continued)

average rate of complete plant recovery (~60%) after liquid nitrogen exposure and rewarming. The number of shoot tips to be cryopreserved per accession was determined with a binomial model that uses as input variables the number of thawed explants (sample size), the expected recovery rate, and the confidence level [17, 18].

2 General Considerations, Supplies, Materials, Culture Media, and Solutions Required for the Cryopreservation Process

Different types of water purification are used in specific parts of this protocol. In the case of culture medium used for in vitro propagation, cold acclimatization, shoot-tip excision, and post-thawing recovery, all solutions are prepared with distilled deionized water. Loading solution (LS), plant vitrification solution 2 (PVS2), and rewarming solution (RS) are prepared with ultrapure water to avoid the presence of ions during the cryoprotection or thawing steps, which can be harmful to the samples. Analytical grade reagents and calibrated/validated equipment should be used during all protocol steps (e.g., pH meter, autoclave, laminar flow chamber, electronic dispensing pump [Tritech Research Inc.]). Purchased pre-prepared full-strength Murashige and Skoog medium (MS), including macronutrients, micronutrients, and vitamins, is used for the preparation of all culture media and solutions (*see Note 1*). Thermolabile compounds (e.g., coconut water) are filter-sterilized and added after autoclaving solutions and allowing them to cool down (35–45 °C). Store chemicals at room temperature (22 ± 3 °C), under refrigeration (5 ± 3 °C) or in the freezer (−15 ± 5 °C), according to what is specified in the Material Safety Data Sheet (MSDS) of each product. Reagents are dissolved using a magnetic stirrer at 600–1000 rpm, depending on the volume and the viscosity of media/solutions that are being prepared.

2.1 Media Preparation

1. Basic medium: 4.43 g/L of commercial Murashige and Skoog (MS) salts with vitamins (Caisson, Smithfield, UT), 25 g/L of sucrose, and 2.8–3.0 g/L of Phytigel® (Merck Sigma-Aldrich, St. Louis, MO) at pH 5.60 ± 0.02 depending on which media is being prepared (propagation or cutting). In a microwave-resistant plastic beaker, dissolve MS salts with vitamins in 600 mL of water, add 25 g of sucrose, and mix. Bring to a final volume of 1000 mL. Adjust pH to 5.60 ± 0.02 with HCl and/or NaOH as needed (*see Note 2*).
2. Propagation medium: Basic medium with 3.0 g of Phytigel. Mix well and dissolve the Phytigel in a microwave (*see Note 3*). Dispense 9.0 ± 0.1 mL per 25 × 150 mm test tube (using an electronic dispensing pump or by manual dispensing). Close test tubes with caps. Autoclave for 20 min at 121 °C and 15 psi. Store at 5 ± 3 °C for maximum 14 days.

3. Cold acclimatization and cutting medium: Basic medium with 2.8 g of Phytigel for standard Petri dishes (100 × 15 mm) and 3.0 g for deep Petri dishes (100 × 25 mm). Prepare in a 1 L Pyrex[®] glass bottle, pour the medium in the bottle, and mix. Autoclave for 20 min at 121 °C and 15 psi. Dispense culture medium in a warm state (~40–50 °C) into sterile deep or standard plastic Petri dishes inside a sterilized laminar flow chamber to reduce possible contaminants. Dispense 40 ± 2 mL per deep Petri dish (cold acclimatization medium) and 25 ± 1 mL per standard Petri dish (cutting medium), using an electronic dispensing pump. Close Petri dishes with sterile lids. Seal deep Petri dishes with parafilm. Store in sterile Petri dish bags at 5 ± 3 °C for a maximum of 14 days.
4. Recovery medium: 4.43 g/L of commercial Murashige and Skoog (MS) salts with vitamins, 25 g/L of sucrose, 20 mL/L of coconut water, 0.1 mg/L of gibberellic acid, 0.4 mg/L of kinetin, 2.8–3.0 g of Phytigel (depending on vessel type/size), pH 5.60 ± 0.02.
 - (a) In a microwave-resistant plastic beaker, dissolve MS salts with vitamins in 600 mL of water, add 25 g of sucrose, and mix. Add water to a volume of 980 mL. Mix well. Adjust pH to 5.60 ± 0.02 with HCl and/or NaOH as needed.
 - (b) Add 3.0 g (for test tubes) or 2.8 g of Phytigel (for Petri dishes) in a Pyrex glass bottle and pour the solution into the bottle. Autoclave culture medium for 20 min (at 121 °C, 15 psi).
 - (c) Place bottle in a laminar flow chamber and let the sterilized culture medium cool down to a temperature of ~35–40 °C. Using a sterile plastic pipette (10 mL), add 20 mL of coconut water stock solution (*see Note 4*). Using a micropipette with sterile tip, add 100 µL of gibberellic acid and 20 µL of kinetin stock solution (*see Note 5*). Gently shake the bottle to get a homogeneous solution.
 - (d) Dispense 25 ± 1 mL of sterile medium per standard Petri dish and 9.0 ± 0.1 mL per 25 × 150 mm glass test tube, using an electronic dispensing pump or by manual dispensing. Close Petri dishes and/or test tubes with sterile lids/caps. Store at 5 ± 3 °C for a maximum of 14 days (in the dark).
5. Loading solution (LS): 4.43 g/L of commercial Murashige and Skoog (MS) salts with vitamins, 136.8/L g sucrose, and 147.4 mL/L glycerol, pH 5.80 ± 0.02.
 - (a) Dissolve the MS salts with vitamins in about 300 mL of Milli-Q water (in a 1000 mL glass beaker). Add 136.8 g of sucrose and mix well.

- (b) Using a glass measuring cylinder, measure 147 mL of glycerol (Merck Sigma-Aldrich, St. Louis, MO) and add it to the solution. As glycerol has a high viscosity, maintain the cylinder on the bench for some time during pouring to ensure an exact glycerol volume in the glass cylinder. It will take time for the glycerol to drop down and stop adhering to the sides of the cylinder due to its viscosity. Mix well.
 - (c) Pour the solution into a glass volumetric flask (1000 mL) and bring to a final volume of 1000 mL with Milli-Q water. Pour the solution back into the beaker. Adjust the pH to 5.80 ± 0.02 with HCl and/or NaOH as needed.
 - (d) Under a laminar flow chamber, filter sterilize LS through a nitrocellulose filter (pore size: $0.22\mu\text{m}$) with a glass fiber pre-filter (pore size: $1.0\mu\text{m}$). Dispense ~ 45 mL of sterilized LS per black centrifuge tube (50 mL). Seal the cap with parafilm and store at 5 ± 3 °C for maximum 14 days.
6. Plant vitrification solution 2 (PVS2): 4.43 g/L of commercial Murashige and Skoog (MS) salts with vitamins, 136.8 g/L of sucrose, 240.0 mL/L of glycerol, 136.4 mL/L of dimethyl sulfoxide (DMSO), 134.8 mL/L of ethylene glycol, pH 5.80 ± 0.02 .
- (a) Dissolve MS salts with vitamins in about 200 mL of Milli-Q water (in a 1000 mL glass beaker). Add 136.8 g of sucrose and mix well.
 - (b) Using a glass measuring cylinder, measure 240.0 mL of glycerol, 136.4 mL of DMSO, and 134.8 mL of ethylene glycol and add them to the solution. Dissolve.
 - (c) Pour the solution into a glass measuring cylinder (1000 mL) and bring to a final volume of 1000 mL with Milli-Q water. Pour the solution back into the beaker. Adjust the pH to 5.80 ± 0.02 with HCl and/or NaOH as needed.
 - (d) Under a laminar flow chamber, filter sterilize PVS2 through a nitrocellulose filter (pore size: $0.22\mu\text{m}$) with glass fiber pre-filter (pore size: $1.0\mu\text{m}$). Dispense ~ 45 mL of sterilized PVS2 per black centrifuge tube (50 mL). Seal the cap with parafilm and store at 5 ± 3 °C for maximum 14 days.
7. Rewarming solution (RS): 4.43 g/L of commercial Murashige and Skoog (MS) salts with vitamins, 205.2 g/L (0.6 M) of sucrose, pH 5.80 ± 0.02 .
- (a) In a 1000 mL glass beaker, dissolve the MS salts with vitamins in about 300 mL of Milli-Q water.
 - (b) Add 205.2 g (= 0.6 M) of sucrose and dissolve.

- (c) Pour the solution into a glass measuring cylinder (1000 mL) and bring to a final volume of 1000 mL with Milli-Q water. Pour the solution back into the beaker. Adjust pH to 5.80 ± 0.02 with HCl and/or NaOH as needed.
- (d) Under a laminar flow chamber, filter sterilize RS through a nitrocellulose filter (pore size: $0.22\mu\text{m}$) with glass fiber pre-filter (pore size: $1.0\mu\text{m}$). Dispense ~ 45 mL of sterilized RS per sterile centrifuge tube (50 mL). Seal the cap with parafilm and store at $5 \pm 3^\circ\text{C}$ for maximum 14 days.

**2.2 Supplies Needed
for Cryopreservation
Process**

1. Flake ice.
2. Liquid nitrogen (*see Note 6*).
3. Aluminum foil (extra heavy duty, thickness: 24–30 μm).
4. Sterile strips of aluminum foil (size: 5 × 20 mm; thickness: 24–30 μm): Strips are cut with scissors and sterilized in standard glass Petri dishes (100 × 15 mm).
5. Forceps for tissue culture (type 1: long straight with fine point, type 2: short and curved [cotton forceps]).
6. Cryoboxes (capacity: 100 cryovials).
7. Sterile cryovials (1.8 mL, internal thread) (Fisher Thermo Scientific, Branchburg, NY).
8. Freezing container (CoolBox™ 30 Systems, BioCision).
9. Ice pan suitable for liquid nitrogen.
10. Parafilm.
11. Plastic wrap.
12. Scalpel holder with blades (Nos. 10 and 11).
13. Sterile bond paper (A5).
14. Sterile filter papers (Grade 2, 10 × 10 mm and 25 × 30 mm).
15. Sterile glass Pasteur pipettes with rubber bulb (1 mL).
16. Sterile glass Petri dishes (100 × 15 mm).
17. Sterile glass screw-top test tubes (15 mL, 70 mm, Ø 20 mm).
18. Dewar flask for liquid nitrogen.
19. Laminar flow chamber.
20. Glass bead sterilizer.
21. Stereoscope.
22. Vortex mixer.
23. Small-, medium-, and high-capacity cryotanks (low pressure; *see Note 7*).
24. High-pressure bulk tank for storage of liquid nitrogen (*see Note 8*).

2.3 Personal Protective Equipment (PPE)

1. Cryo-apron.
2. Cryogenic gloves.
3. Face shield.
4. Safety goggles and glasses.
5. Transfer hose for liquid nitrogen.

3 Methods

As cryopreservation is a labor-intensive, multistep process (Fig. 1) for introducing plant material into the cryobank, it is recommended to cryopreserve only pathogen-free (e.g., virus) and identity verified accessions (also known as true-to-type material). Establishment of virus-clean plant material and identity verification schemes will avoid conserving duplicates or non-true-to-type accessions. If technically possible, all steps of the *in vitro* and cryopreservation processes should be recorded in a real-time database using barcode labels as input (handwriting of labels is often a potential source of human error and may cause misidentification of accessions). Furthermore, ensure that all staff are highly trained before using and handling liquid nitrogen and associated equipment. Use only vessels and tanks that have been especially designed for holding liquid nitrogen. Wear adequate personal protective equipment (PPE) when using or handling liquid nitrogen (*see Note 9*).

Finally, it is also important to establish clear threshold values and standards for minimum acceptability of full plant recovery rates. The threshold varies depending on the plant species, number of stored and thawed samples, and expected recovery rate and probability to recover plants [15, 16]. At CIP, a minimum acceptable plant recovery rate of 30% was established for transferring accessions to the cryobank for long-term preservation. This recovery assessment is based on a sample size of 30 shoot tips assessed from a total of 150 cryopreserved shoot tips (per accession). Accessions that show a recovery rate of 20–30% in the first cryo-run require a second additional run, in order to increase the total number of stored samples. Accessions that show less than 20% of recovery rate are discarded and not transferred to the cryobank and later cryopreserved with an alternative protocol [5]. Anything greater than 30% is stored in the cryobank for a long term.

3.1 *In Vitro* Propagation Cycles

1. Always follow good laboratory practices for *in vitro* propagation (*see Note 10*).
2. Propagate stem segments of *in vitro* potato plants onto propagation medium (*see Subheading 2.1*).
3. Place 5–6 stem segments per 25 × 150 mm test tube, with one to two buds per segment (Fig. 2) (*see Note 11*).



Fig. 2 In vitro propagation of potato. Planting of stem segments, with 1–2 buds, on basic culture medium contained in 25 × 150 mm glass test tubes. Per test tube place 5–6 stem segments

4. Close test tubes with sterile caps.
5. Label test tubes with an accession identifier and date of propagation.
6. Incubate in vitro plants for 2–4 weeks at a temperature of 20 ± 2 °C with a light intensity of $96 \pm 12 \mu\text{mol}/\text{m}^2/\text{s}$. As a light source, use cold daylight fluorescent tubes (36 W), with a photoperiod of 16-h light and 8-h darkness. The incubation period depends on genotype and growth rate.
7. Perform a second and third propagation cycle until a total number of 14 test tubes has been obtained (~70 in vitro plants).

3.2 Cold Acclimatization of Shoot-Tip Donor Plants

1. Place stem segments with a single axillary bud onto the cold acclimatization medium. Place 80–90 stem segments per deep Petri dish. Propagate a total number of two Petri dishes per accession (~160–180 plants) (Fig. 3).
2. Incubate Petri dishes for 7–10 days at 20 ± 2 °C and light intensity of $96 \pm 10 \mu\text{mol}/\text{m}^2/\text{s}$, followed by 10–25 days at 7 ± 1 °C and light intensity of $15 \pm 5 \mu\text{mol}/\text{m}^2/\text{s}$ (cold chamber) (*see Note 12*).

3.3 Excision of Potato Shoot Tips

1. Distribute 15 small square pieces of sterile filter paper (10 × 10 mm) on the surface of sterile cutting medium.

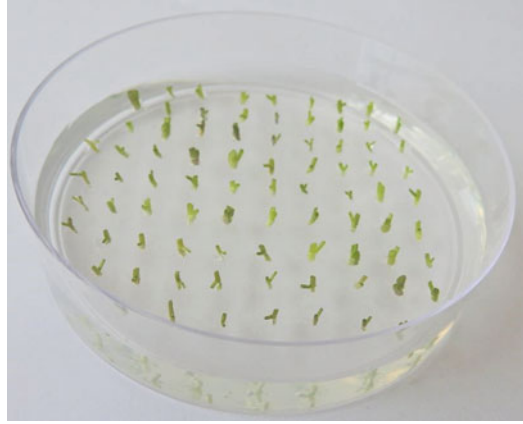


Fig. 3 Deep Petri dish (25 × 100 mm) containing ~80 potato stem segments with a single axillary bud. Stem segments are pre-cultured for 7–10 days at 20 ± 2 °C, followed by 10–25 days at 7 ± 1 °C (cold hardening). The specific incubation period varies depending on the genotype and physiological status of the accession. After 17–35 days, the shoot tips are excised from these *in vitro* plants, treated with cryoprotectant solutions, and fast frozen in liquid nitrogen

2. Use long tissue culture forceps and scalpel No. 11 excise shoot tips (*see Note 13*) from 17- to 35-day-old *in vitro* plants coming from Subheading 3.2 under the stereoscope (*see Note 14*).
3. Place excised shoot tips individually onto the filter papers of the cutting medium. First place one single shoot tip onto each of the filter papers, then place the second shoot tip onto each of the filter papers, then the third, and so on. At the end of this process, each filter paper will hold 10 shoot tips. Per accession, excise 150 shoot tips (the shoot tips were placed on a total of 15 filter papers).

3.4 Preparative Steps for Cryopreservation

1. Using a permanent marker write accession code and freezing date on the cryotubes (temporary label).
2. Print cryogenic barcode labels and place them onto the handwritten identification (double identification in case the label gets damaged) (*see Note 15*).
3. Label and set up glass tubes with screw-top lids (15 mL) for the cryoprotectant treatment (*see Note 16*).
4. Set up two Pasteur pipettes to handle the cryoprotectant solutions (LS and PVS2), shoot-tip absorption, and droplet formation (*see Note 17*).
5. Homogenize LS with a vortex mixer.
6. Using a Pasteur pipette, dispense ~1.0 mL of LS per sterile screw-top test tube (15 mL).

7. Fill ice pan with flake ice.
8. Place a 50 mL centrifuge tube with sterile PVS2 on ice (0 °C) at a depth of $\sim 3/4$ of the tube.

**3.5 Treatment
with Cryoprotectant
Solutions
(LS and PVS2)**

1. Using long tissue culture forceps (23 cm), place filter paper with shoot tips into LS (Fig. 4). Discharge shoot tips from filter paper. Remove filter paper from the test tube (*see Note 18*). Treat shoot tips with LS for 20 min (at room temperature).
2. Using a Pasteur pipette remove LS from the tube (*see Note 19*) and replace it with ~ 1.0 mL of ice-cold PVS2 (*see Note 20*).
3. Close test tube with sterile cap and verify that all shoot tips are submerged in PVS2 (use white absorbent paper as background).
4. Treat the shoot tips with PVS2 for 50 min by keeping $3/4$ of the tube on ice (Fig. 5).

**3.6 Freezing
in Liquid Nitrogen**

1. Prepare aluminum foil strips (*see Note 21*) and freezing container (CoolBox™ 30 Systems) (*see Note 22*).
2. Place labeled cryovials in the holder of freezing container (*see Note 23*).
3. Using Pasteur pipette, absorb shoot tips from PVS2, place them in a small volume of PVS2 (droplet of $\sim 20\text{--}25\mu\text{L}$) onto the aluminum foil strip (*see Note 24*) (Figs. 6 and 7), quickly plunge the strip into liquid nitrogen (“fast freezing”), and transfer it to a cryovial (*see Note 25*) (Fig. 8).
4. Close cryovials with sterile caps (*see Note 26*).
5. Store cryovials in a cryotank suitable for laboratory use (*see Note 27*). Cryovials are placed on aluminum canes (cryovial holders) for temporary storage (*see Note 28*) (Fig. 9).

**3.7 Transitory
Storage**

1. Label and UV sterilize cryoboxes (capacity: 100 vials) and two liquid nitrogen pans for 15 min (*see Note 29*).
2. Fill pans with liquid nitrogen in a laminar flow chamber (LFC), at a height of approximately 3–5 cm (*see Note 30*).
3. Place two empty cryoboxes in one of the pans (in LFC).
4. Move laboratory and transitory storage tank next to LFC (*see Note 31*).
5. Place cryo-canes holding the cryovials to be transferred in the second pan (*see Note 32*).
6. Using sharp-point pliers, quickly transfer cryovials from aluminum canes to cryoboxes (*see Note 33*). When the cryobox is full, close it with its sterile lid, and transfer it to the transitory storage tank (*see Note 34*).



Fig. 4 A piece of filter paper (1 × 1 cm) with 10 potato shoot tips is introduced to a screw-top glass tube (70 × 20 mm), containing ~1.0 mL of loading solution (LS). Shoot tips are treated for 20 min with LS (at room temperature). After 20 min, LS is replaced with ~1.0 mL of ice-cooled plant vitrification solution 2 (PVS2)



Fig. 5 Shoot tips are treated with PVS2 for 50 min (at 0 °C)

3.8 Thawing of Shoot Tips in Rewarming Solution (RS)

1. Set up sterile screw-top glass tubes (15 mL) for thawing of the control samples and set up a Pasteur pipette to handle the rewarming solution (RS) and shoot tips during the thawing process (*see Note 35*).
2. Dispense ~2.0 mL of sterile RS per sterile screw-top test tube, label tubes, and place three square pieces of filter paper on recovery medium in sterile plastic Petri dishes (*see Note 36*).
3. Set up freezing container and fill it with liquid nitrogen (as described in **Note 22**). Using sharp-point pliers, transfer



Fig. 6 Ten potato shoot tips contained in a small volume of PVS2 in the tip of a Pasteur pipette, before it is placed on the aluminum foil strip



Fig. 7 Shoot tips contained in a droplet of PVS2 (~20–25 μ L) that was placed onto a small aluminum foil strip (5 \times 20 mm, with a 3 mm fold). The aluminum foil strip (with shoot tips on it) is then plunged into liquid nitrogen for quick freezing

cryovials to be thawed from the laboratory cryotank to the cryovial support of the freezing container.

4. Transfer aluminum foil strip with shoot tips individually to tubes with RS. Rewarm shoot tips in RS for 20 min at room temperature (*see Note 37*) (Fig. 10).
5. Place three large filter papers (~30 \times 35 mm) onto a stack of 3–6 sterile paper bond sheets. Distribute three small filter papers (~10 \times 10 mm) on each of the large filter papers (*see Note 38*).

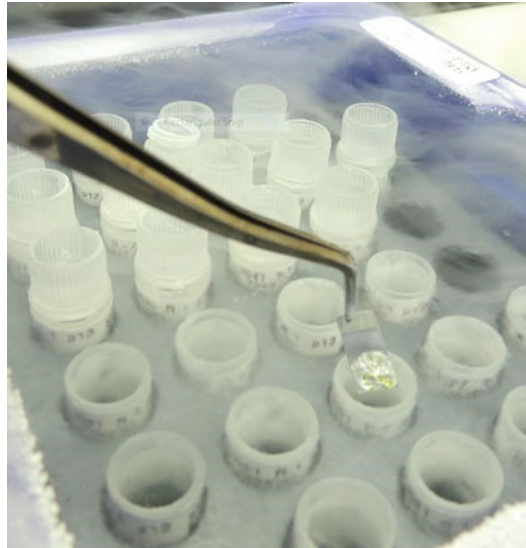


Fig. 8 Freezing of aluminum foil strip with ten shoot tips on it in liquid nitrogen. The strip is plunged quickly into liquid nitrogen (“fast freezing”). A single strip is placed per cryovial (= 10 shoot tips)

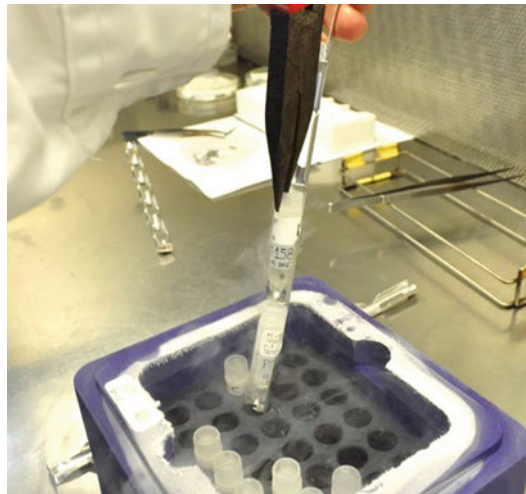


Fig. 9 Using sharp-point pliers or cotton forceps, capped cryovials are quickly transferred from the freezing container to an aluminum can that was previously precooled in liquid nitrogen

6. Using the Pasteur pipette, absorb shoot tips from RS and place them for approximately 1–3 min on the small sterile filter papers (to drain excess of RS solution) (Fig. 11).



Fig. 10 Thawing process. Aluminum foil strip with ten cryopreserved potato shoot tips (in a vitrified droplet of PVS2) is quickly introduced into a vial with ~2.0 mL of rewarming solution (RS). Shoot tips are treated for 20 min with RS

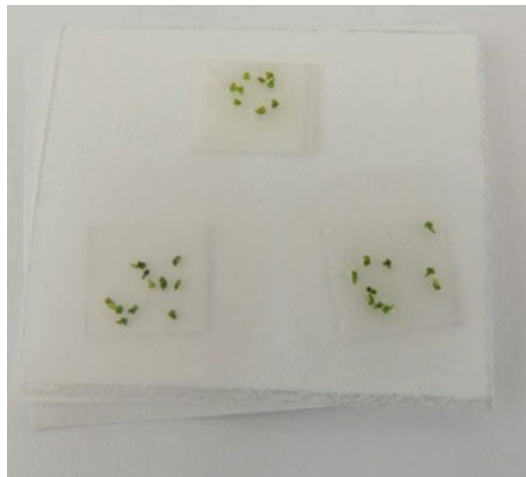


Fig. 11 Concluded RS treatment shoot tips are absorbed with a Pasteur pipette and placed for 1–3 min onto small filter papers (1 × 1 cm) for draining of excess of RS

7. Transfer shoot tips to recovery media (*see Note 39*). Each of the three filter papers of the recovery medium will hold ten shoot tips (coming from each of the individually thawed aluminum foil strips) (Fig. 12).
8. Seal Petri dishes with parafilm and label dish.

3.9 Recovery Cycle

1. Wrap Petri dishes (from the end of Subheading 3.7) in aluminum foil (to ensure that the material stays in the dark).
2. Incubate shoot tips for 9 days on recovery medium at 20 ± 1 °C, wrapped in foil.
3. Transfer shoot tips from each filter paper to a separate Petri dish with fresh sterile recovery medium, using tissue culture (23 cm) or cotton forceps. At this stage, the shoot tips are

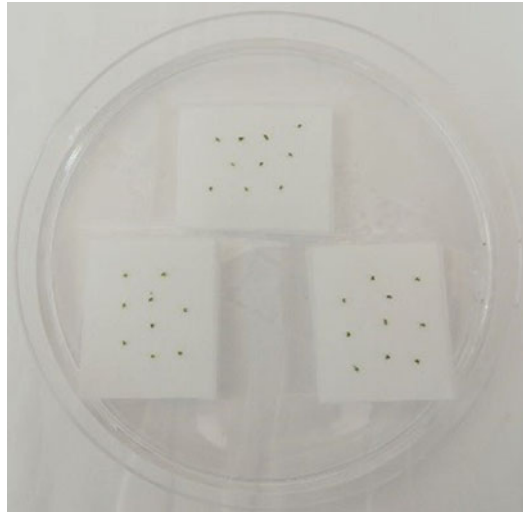


Fig. 12 Each small filter paper, with the shoot tips facing down, is slid over one of the large filter papers that is supported onto the recovery medium, such that the shoot tips are unloaded. The shoot tips are spread evenly over the large filter paper

placed directly onto the culture medium without filter paper support. Place them right side up with the meristem pointing upwards to facilitate proper growth. Seal Petri dish with parafilm and label.

4. Incubate shoot tips for 4 days at 20 ± 1 °C, under diffuse light (loosely place a sheet of aluminum foil on top of the Petri dishes) with a photoperiod of 16-h light/8-h darkness.
5. After 4 days in diffuse light, remove the foil from the Petri dishes and incubate shoot tips for ~18 days under normal environmental conditions with a temperature of 20 ± 2 °C, photoperiod of 16-h light/8-h darkness, and $96 \pm 12 \mu\text{mol}/\text{m}^2/\text{s}$ of light intensity, supplied by cold-day fluorescent tubes.
6. 30–35 days after thawing, transfer surviving and recovered shoot tips and/or plantlets from Petri dishes to fresh sterile recovery medium in 25×150 mm test tubes. Close test tubes, label, and seal with plastic wrap (*see Note 40*).
7. Incubate the plantlets for ~30 days at a temperature of 20 ± 2 °C and $96 \pm 12 \mu\text{mol}/\text{m}^2/\text{s}$ of light intensity, supplied by fluorescent tubes (cold daylight) with a photoperiod of 16-h light/8-h darkness.

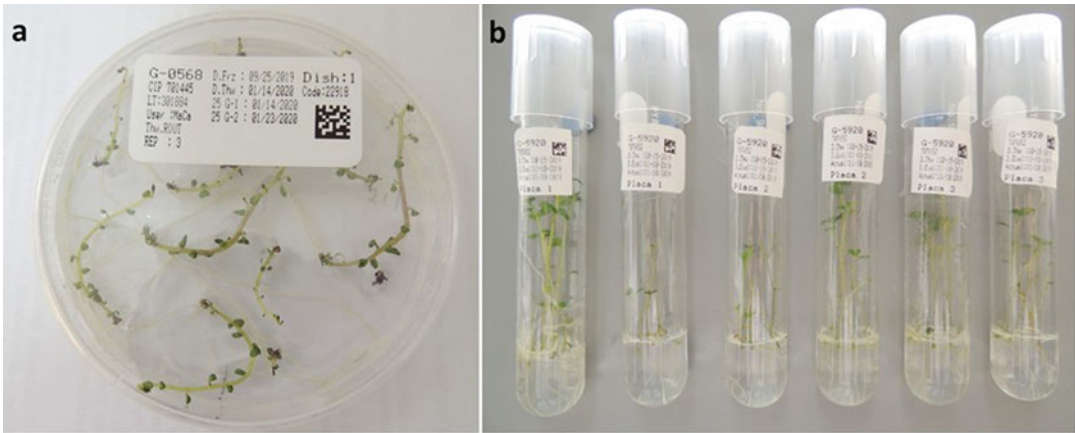


Fig. 13 (a) Potato plants in Petri dishes, 30 days after thawing (exposed to liquid nitrogen). During the first 9 days of the recovery cycle the shoot tips are cultured in darkness, followed by ~21 days under normal light conditions. Samples of each thawed cryovial (= 10 shoot tips) are placed in a separate Petri dish. (b) In vitro potato plants, 60 days after thawing (final assessment). After 30 days in Petri dishes, fully recovered and survived plants are transferred to 25 × 150 mm test tubes. A maximum of five plants is placed in each test tube. Generally, the plants of each Petri dish (of the 30-day assessment) end up in two test tubes (max. 10 plants)

3.10 Assessment of Survival and Recovery Rates of Liquid Nitrogen-Treated Shoot Tips

1. Assess survival and recovery rates at 30–35 and 60–65 days after thawing.
2. In the first assessment which occurs 30–35 days after thawing, a shoot tip is classified as surviving if it shows green tissue, and as recovered if a shoot grows directly or laterally from the meristematic dome, without intermediate callus formation (Fig. 13a).
3. Perform the final assessment of survival and determine recovery rates, 60–65 days after thawing (in test tubes). Only samples that have developed into a complete, normal-appearing in vitro plantlet are counted as recovered (i.e., plants with elongated stem, functional apex, leaves, and roots) (Figs. 13b and 14a, b). Tissue with any type of growth and/or plants with abnormal features (deformation, lack of development, vitrification, etc.) are recorded as survived or dead (*see Note 41*) (Fig. 14c–g).

3.11 Discarding of Accessions

1. Accessions that did not fulfill the minimum recovery rate threshold (<20%) or show signs of bacterial/fungal contamination (human error) (Fig. 15a–c, also *see Note 42*) are discarded from the transitory tank and not transferred to the cryobank/safety backup tanks.
2. Transfer the cryobox containing the accession(s) to be discarded into a pan filled with approximately 3–5 cm of liquid nitrogen that was previously sterilized by UV light (in LFC).
3. Discard cryovials of the accession to be eliminated from the transitory cryobox (*see Note 43*).

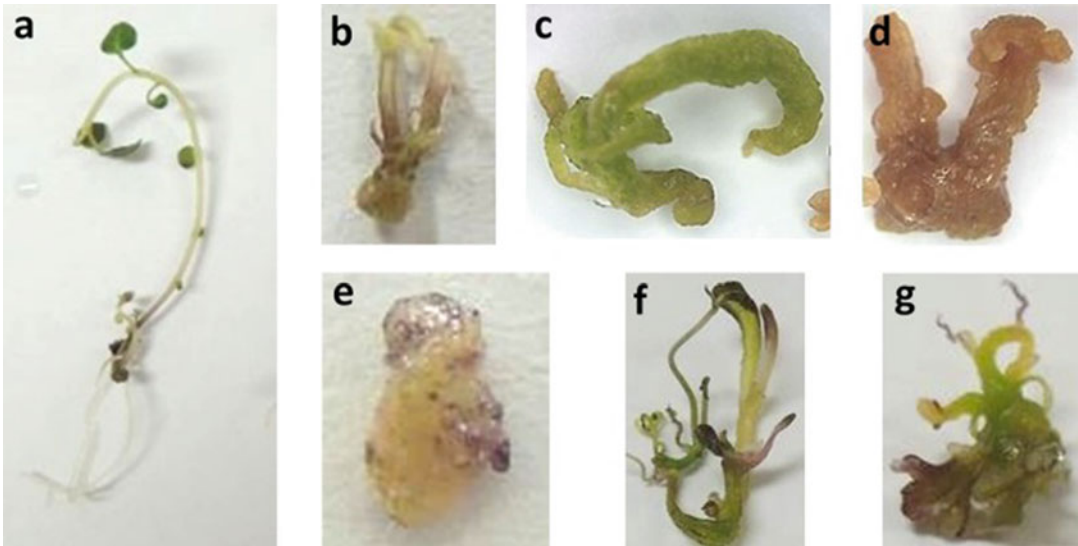


Fig. 14 Assessment criteria for tissue or plant survival and plant recovery, 60 days after thawing. Only a and b plants were classified as recovered. (a) Completely recovered plant (normal aspect with elongated shoot, leaves, functional apex, and roots), (b) partially recovered plant (requires a subculture cycle to confirm if it will develop into a complete plant), (c) only leaf formation without functional apex (survived), (d) phenolized sample (dead), (e) undifferentiated growth or callus (survived), (f) vitrified plant (survived), (g) deformed plant (survived)

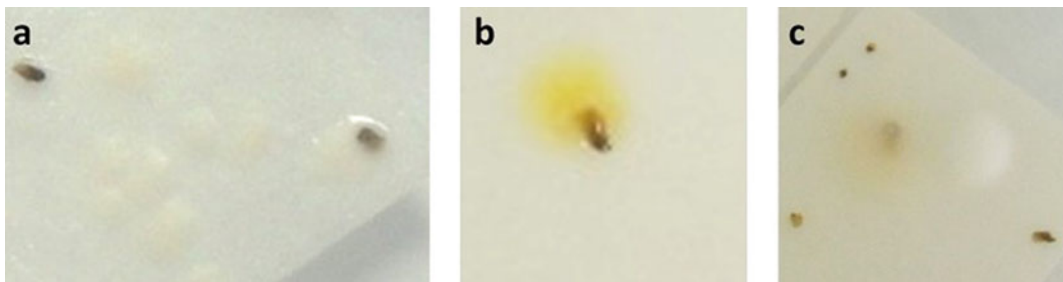


Fig. 15 Bacterial or fungal contamination of thawed shoot tips (9 days after thawing). If one or more individual shoot tips are contaminated, generally the problem has occurred during shoot tip transfer. If the complete filter paper of the recovery medium is contaminated, probably the contamination has happened during shoot-tip excision, treatment with cryoprotectant, and thawing solution. (a) Bacterial contamination on the filter paper of the recovery medium (white milky colony), (b) bacterial contamination of shoot tip (yellow colony), (c) fungal contamination of shoot tip and filter paper (wooly white-gray aspect)

3.12 Final Transfer to Cryobank and Safety Backup Tanks

1. Label and UV sterilize cryoboxes for final storage (capacity: 100 vials per cryobox) (*see Note 44*).
2. UV sterilize two liquid nitrogen pans (*see Note 29*).
3. Fill pans with liquid nitrogen to a height of approx. 3–5 cm (in LFC) (*see Note 30*).

4. Transfer two cryoboxes from the transitory tank to the first pan. These boxes contain the accessions that will be transferred to the cryoboxes of the cryobank and safety backup tank.
5. Place two previously UV-sterilized cryoboxes into the second pan. These are the cryoboxes, to which the cryovials will be transferred. One of the boxes will later be transferred to the cryobank tank and the other one to the safety backup tank.
6. Transfer cryovials of accessions that fulfill the minimum recovery and quality criteria from the transitory to the cryobank and safety backup boxes (using tissue culture or cotton forceps or sharp-point pliers) (*see Note 45*).
7. When a cryobank or safety backup box is full, close it with its lid, and transfer it to a small cryobox-based laboratory tank.
8. Move laboratory tank next to the cryobank/safety backup tanks (*see Note 46*).
9. Transfer cryoboxes from laboratory tank to the cryobank and safety backup tanks (*see Note 47*).

3.13 Monitoring of Liquid Nitrogen Level and Filling of Cryotanks

1. Periodically record liquid nitrogen level of all cryotanks (*see Note 48*).
2. Fill cryotanks periodically with liquid nitrogen (the liquid nitrogen level must always be maintained above the specific minimum level of the tank) (*see Note 49*).

4 Notes

1. The usage of pre-prepared commercial in vitro culture media, instead of preparing individual stock solutions for macronutrients, micronutrients, and other additives, results in a substantial time saving and enhanced quality control by reducing the risk of human error during media preparation. The MS media (with vitamins) has the following composition: 1650 mg/L of ammonium nitrate, 6.2 mg/L of boric acid, 332.2 mg/L of calcium chloride (anhydrous), 0.025 mg/L of cobalt chloride (hexahydrate), 0.025 mg/L of cupric sulfate (pentahydrate), 37.26 mg/L of ethylenediaminetetraacetic acid (EDTA) (disodium salt, dihydrate), 27.8 mg/L of ferrous sulfate (heptahydrate), 2 mg/L of glycine, 180.7 mg/L of magnesium sulfate (anhydrous), 16.9 mg/L of manganese sulfate (monohydrate), 0.25 mg/L of molybdic acid sodium salt (dihydrate), 100 mg/L of myoinositol, 0.5 mg/L of nicotinic acid, 0.83 mg/L of potassium iodide, 1900 mg/L of potassium nitrate, 170 mg/L of potassium phosphate (monobasic, anhydrous), 0.5 mg/L of pyridoxine (hydrochloride), 0.1 mg/L of thiamine (hydrochloride), and 8.6 mg/L of zinc sulfate (heptahydrate).

2. In general, NaOH (1 M) and HCl (1 M) are used for pH adjustment. To increase and decrease the pH, add drop(s) of NaOH and HCl to a solution, respectively. For solutions with a final volume of less than 500 mL, it is recommended to use more diluted solutions of NaOH and HCl for pH adjustment (e.g., 0.5 M). To avoid diluting highly concentrated acids or sodium hydroxide which can be a health and safety risk, purchase them in an already diluted form (1 M) (Merck Sigma-Aldrich, St. Louis, MO). Start the pH adjustment by adding 3–5 drops of HCl or NaOH at once while stirring the solution. Wait for 2–3 min for the pH to stabilize and then continue adding smaller volumes as needed. When the pH adjustment gets close to the required value (e.g., 5.8), dilute some drops of HCl or NaOH in distilled water and use this solution for the final pH adjustment.
3. Use a microwave oven to dissolve the gelling agents of solid culture media that do not contain any thermolabile compounds. When the gelling agent has started to dissolve (before boiling), take the beaker out of the microwave and homogenize with a magnetic stirrer to avoid the formation of agglomerates. Next, place it back in the microwave and heat until boiling and mix. The gelling agent is dissolved completely when the solution changes from cloudy (milky) to a transparent/clear solution. For instance, to prepare 1 L of culture media, heat the culture medium for 7–8 min in the microwave, homogenize the solution on a magnetic stirrer, and then continue heating for 3–4 min more. The heating time will be approximately doubled for a 2 L solution. When a dispensing pump is used for pouring the culture media, the beaker containing the media is placed on a magnetic stirrer (to ensure a homogeneous composition in all vessels).
4. Coconut water is a highly complex solution that contains minerals, vitamins, sugars, amino acids, hormones, and proteins. Use commercially available tissue culture-tested coconut water (e.g., Merck Sigma-Aldrich, Product Code: C5915) to guarantee homogeneity and purity of the coconut water. Filter sterilize coconut water in the laminar flow chamber through a nitrocellulose filter (pore size: 0.22 μ m) with glass fiber pre-filter (pore size: 1.0 μ m). Dispense 20–30 mL of coconut water per dark sterile plastic container. Close the container with sterile cap and seal with parafilm. Store coconut water at -20 ± 5 °C (in the dark). Before using, defrost coconut water and dispense the required volume with a sterile pipette. Defrost coconut water only two times (avoid frequent thawing and refreezing).
5. The gibberellic acid and kinetin stock solutions are prepared at 1000 ppm and 2000 ppm, respectively. Weigh the reagent in a

small beaker, dissolve it with 2–3 drops of NaOH (1 M) (for gibberellic acid) and 2–3 drops of NaOH (1 M) or ethanol (96%) (for kinetin), then add distilled water to its final volume, and mix. When plant growth regulator stock solutions are prepared, it is recommendable to use HCl (0.1–1.0 N) to dissolve basic reagents and NaOH/KOH (0.1–1.0 N) to dissolve acidic agents. Hormone stock solutions are filter sterilized in the laminar flow chamber through a nitrocellulose filter (pore size: 0.22 μ m) with glass fiber pre-filter (pore size: 1.0 μ m). The hormone stock solutions are labeled and stored in screw-top test tubes (in the dark) at 5 ± 3 °C for a maximum of 4 weeks, or at -20 ± 5 °C (in darkness) for a maximum of 1 year.

6. Liquid nitrogen of high purity (min 99.0%) should be employed for all protocol steps. When producing liquid nitrogen in-house with a generator, ensure adequate maintenance of all components. CIP's liquid nitrogen generator has four principle sections to be considered: liquefier section (e.g., cold head), refrigeration section (e.g., helium compressor, adsorber), pressure swing adsorption section (PSA) (includes feed air and nitrogen product filters, valves, hoses, manometers), and air compressor section (filters, sealings, lubrication, manometers, etc.).
7. Different types and capacities of cryotanks are available. Tanks for storage of cryovials can be equipped with cryoboxes or cryo-cane holders. Use cryo-cane-based tanks for initial storage, and cryobox-based tanks for transitory, final, and safety backup storage. Large-capacity tanks for final storage generally have space for storage of 10,000 to ~100,000 cryovials. The CIP tanks have a capacity of ~20,000 cryovials, which permits conservation of ~1800 accessions per tank (with one single cryo-run). It is recommended to equip the cryotanks for long-term storage with an electronic liquid nitrogen monitor and temperature probes to manage and document the liquid nitrogen level, access, alarm, temperature settings, filling cycle (automatic or semiautomatic), and other features. Additionally, the liquid nitrogen monitor should be connected to a backup power generator/battery (to permit continuous data record and operation in the case of energy breakdowns). Ensure that the lid of the cryotank permits escape of evaporating gaseous nitrogen from the inside of the tank.
8. High-pressure tanks are used to fill low-pressure tanks and liquid nitrogen Dewars. High-pressure tanks are generally equipped with four types of valves: liquid, venting, pressure buildup, and pressure escape (security valve). When filling high-pressure tanks, first open the venting valve to relieve the remaining pressure of the tank (up to 0–0.5 psi), leave the venting valve open, connect the filling hose (which is

connected to the bulk tank of the liquid nitrogen generator), open the liquid valve of the high-pressure tank, and then start the filling process from the generator. Fill the tank to a maximum of 80% of its capacity. Stop the filling process on the generator and close the liquid and venting valves of the high-pressure tank. Disconnect the hose from the filling valve and open the pressure buildup valve, to increase the internal tank pressure in a short time period (~30 min) to a value of 10–23 psi which allows efficient pouring of liquid nitrogen. Close the pressure buildup valve. Connect a hose to the connector of the liquid valve to pour liquid nitrogen out of the tank (e.g., for filling of low-pressure tanks). If the high-pressure tank is not equipped with a pressure-build valve, it may take ~24 h until the internal tank pressure is high enough to permit pouring of liquid nitrogen.

9. Only use liquid nitrogen in well-ventilated spaces because 1 L of liquid nitrogen gives 0.7 m³ of nitrogen gas which will rapidly displace oxygen in air. Rooms in which large volumes of liquid nitrogen are handled or stored should be equipped with an electronic oxygen alarm. Cryogenic gloves and aprons, goggles, or face shield (not safety glasses) are always to be worn when handling small volumes of liquid nitrogen (for example when it is transferred from small Dewars (capacity of 1–4 L) to freezing containers). When handling larger volumes of liquid nitrogen (for example to fill a large-capacity cryotank), wear full-length cryogenic apron, face shield, and cryogenic gloves. Do not allow liquid nitrogen to touch any part of your body or become trapped in clothing near the skin. Do not store liquid nitrogen in any container with tight-fitting lid and only use fittings that have been designed specifically for use with liquid nitrogen. Never dip a hollow tube into liquid nitrogen, as it may spurt liquid nitrogen. Always fill warm Dewars slowly to reduce temperature shock effects and to minimize splashing. Do not fill cylinders and tanks to more than 80% capacity, since expansion of gases during warming may cause excessive pressure building. Crack open the lids of all cryovials immediately upon removal from liquid nitrogen to prevent pressure from building up in the vial (explosion can happen when liquid nitrogen leaks into a poorly sealed vial and rapidly expands after the vial is removed from liquid nitrogen).
10. Use long tissue culture forceps (23 cm) and scalpel with blade No. 10 to carry out *in vitro* propagation. Place the plants on top of a stack of 2–3 sterile bond paper sheets. Remove leaves and roots, cut segments of stems (with 1–2 buds), and place them in containers with sterile propagation medium. Exceptionally, segments with three buds can be used, in case the distance between nodes is very short [19]. Use only the

upper two-thirds of the plant for propagation and discard the lower third of the plant as basal buds frequently show bad shoot growth. Replace the stack of paper sheets after each accession to avoid cross contamination. When labeling test tubes and deep Petri dishes, first stick the label onto a piece of plastic wrap and then label the container by wrapping the plastic wrap around the lid/cap of the test tube. This prevents the label from sticking directly on the container and its subsequent removal is simpler. The junction between the tube and cap/lid should be sealed with one or two layers of plastic wrap or parafilm to prevent mite infection. Place the label from the original container onto the new Petri dish or test tube where the material is being transferred to, as a control label along with the newly printed label. This helps to track correct labeling in each multiplication cycle. Contamination (fungal and bacterial) along with plant growth and development (shoot growth and rooting) is assessed 5–7 and 10–14 days after propagation. Test tubes with signs of contamination or bad growth of plants are discarded, and the propagation is restarted with a new, clean growing stock of plants.

11. A total number of three tubes is obtained in the first propagation cycle when the propagation has been started from one single test tube with five *in vitro* potato plants. The original *in vitro* plants came from the medium-term storage (MTS) chamber, where the *in vitro* plants can be stored from 12 to 36 months (genotype dependent) at 7 ± 1 °C.
12. The incubation periods can vary depending on genotype, physiological status, and plant growth. *In vitro* rooms are equipped with cold daylight fluorescent tubes (36 W), and a photoperiod of 16-h light/8-h darkness.
13. The shoot tip should contain 2–4 leaf primordia, and have a length of 0.8–1.2 mm and a width of 0.3–0.7 mm (at the height of the meristem). Length and width of shoot tips vary depending on the potato genotype (Fig. 16).
14. The optimal age for excision varies between accessions, depending on their growth and development. Use a magnification factor of 10× during shoot-tip excision. Use a stack of 2–3 sterile bond paper (A5) as a cutting surface for the shoot-tip excision and replace it periodically (after each batch of processed plants). Excision is done in groups of 10–30 plants (depending on the experience and speed of the person). After each processed group, replace the paper stack to avoid cross contamination. During stereoscope usage, close your eyes and/or focus on an item far away every 15 min to reduce eye strain.



Fig. 16 Potato shoot tip of length of 0.8–1.2 mm and width of 0.3–0.7 mm (genotype dependent)

15. One of the cryovials will contain 30 shoot tips as control samples (3 aluminum foil strips with 10 shoot tips on each). This cryovial will be thawed and regrown for the assessment of the survival and plant recovery rates. The other cryovials will contain 10 shoot tips each and will be transferred to the cryobank and safety-backup tanks, if the accession accomplishes the minimum criteria for recovery and does not show any signs of bacterial or fungal contamination.
16. Place small labels (1 × 1 cm) onto the caps of sterile screw-top test tubes (15 mL) that will be used for the cryoprotectant treatment with loading solution (LS) and plant vitrification solution 2 (PVS2). Maintain one row free of tubes between different accessions in the rack to help reduce the chance of misplacement between accessions. The labels contain the following information: accession identifier, “LS:” and “PVS2:” Place tubes in a test tube rack.
17. Disinfect rubber bulbs (1 mL) with 70% alcohol. Place rubber bulbs on two sterile Pasteur pipettes. One of the pipettes is used for pipetting LS and the other for PVS2. It is recommended that the opening of the LS pipette is of an adequate size that does not allow the shoot tips to pass (<0.8 mm), while the opening of the PVS2 pipette must have a dimension that allows the absorption and expulsion of the shoot tips (>1.2 mm). Place pipettes individually in sterile vessels (e.g., screw-top test tubes). If the pipette tip comes into contact with a non-sterile object or an inappropriate solution is absorbed, discard it (and later heat sterilize it for reuse) and immediately

replace it with a new sterile Pasteur pipette of the same type (with the tip openings described for either LS or PVS2).

18. During introduction of shoot tips into LS, hold the test tube slightly at an angle. Immerse filter paper with a gentle movement in the LS solution to prevent shoot tips from sticking to the test tube wall or not detaching from the filter paper. Remove filter paper from the test tube. Shake gently. Visually, verify that all shoot tips are submerged in LS. Using a marker and a temporary label, record the end time of LS treatment on the cap and monitor treatment time with a digital timer. If shoot tips get stuck to the test tube, incline the tube in such a way that shoot tips get detached and make contact with the LS solution. Alternatively, absorb some LS with the Pasteur pipette and detach the shoot tips expelling the LS. Pause for 1–3 min between each filter paper inserted (depends on operator experience) to avoid rushing and mistakes generated during the PVS2 treatment and droplet formation.
19. When the LS treatment has concluded, siphon off the remaining LS solution from the tube with the Pasteur pipette, so that shoot tips remain at the bottom of the tube (without LS solution). It is recommended to keep the tube upright and introduce the pipette almost flush with the bottom (at such depth that shoot tips cannot enter through the pipette opening). Visualization of shoot tips is facilitated by placing a white absorbent paper behind the test tube. Discard absorbed LS into a container. It is recommended to use a sterile container for discarding to avoid any risk of cross contamination (if the glass pipette gets in contact with the rim of a non-sterile container it may get contaminated).
20. Homogenize PVS2 in a light-resistant centrifuge tube (50 mL) using a vortex mixer (DMSO is light sensitive). Using a marker record the end time of the PVS2 treatment on the test tube cap. Monitor PVS2 treatment time with a digital timer.
21. While shoot tips are treated with PVS2, place small sterile aluminum strips (5 × 20 mm) in a sterile disposable Petri dish lid. Using scalpel holder, tissue culture, or cotton forceps fold aluminum strips slightly at one end (fold of approximately 3 mm at 90° angle) (Figs. 1b and 7).
22. Approximately 10–15 min before PVS2 treatment has finished, disinfect the freezing container and its cryovial holder using a spray bottle with 70% alcohol solution (in LFC). Let the excess alcohol drain or evaporate, placing support and container upside down on a paper towel. Place cryovial support block into the freezing container. Carefully pour liquid nitrogen from the 4 L Dewar flask into the freezing container. At the beginning, a large amount of liquid nitrogen evaporates. The container should be refilled 3–5 times until the freezing

container has cooled sufficiently (no more bubble formation is observed on the surface of the liquid nitrogen).

23. Uncap cryovials and introduce them to the freezing container. Let cryovials float on the surface of the liquid nitrogen. Never introduce cryovials directly to the holes of the cryovial holder, because the sudden change of temperature can splash liquid nitrogen toward the face of the operator. The cryovials must first have cooled down sufficiently by swimming on the liquid nitrogen surface, before placing them with forceps in the corresponding holes of the support block. The liquid nitrogen level should always be ~1–1.5 cm above the openings for the cryovials in the support block. Identify each accession with a cryogenic label on the freezing container. Leave free space between different accessions.
24. When absorbing the shoot tips with the Pasteur pipette from the PVS2 solution, place the tip of the pipette at the bottom of the tube and slowly eject PVS2 from the pipette. Shoot tips are contained in a very small volume of PVS2 (~20–25 μ L) on the tip of the pipette. Using cotton forceps, hold the aluminum strip by its fold and place shoot tips together with the PVS2 droplet onto the central part of the strip (Figs. 1b and 7). The drop must contain all shoot tips and have a semispherical shape. If the PVS2 droplet comes in contact with the forceps holding the strip, reabsorb shoot tips and PVS2, change forceps, and place them onto a new strip. If PVS2 comes in contact with the forceps, the strip sticks to the forceps during freezing in liquid nitrogen, and it may cause the shoot tips to fall or damage them.
25. Move the strip only below the surface of liquid nitrogen. Place a single strip per storage cryovial (= 10 shoot tips), except for the control vial that contains 3 strips (= 30 shoot tips), which are thawed later to obtain the initial recovery rate. Place the caps of the PVS2 tubes in the same order in the LFC as the cryovials are placed in the freezing container (operative quality control). Verify matching of accession identifiers on cryovials and test tube caps (PVS2) and their correlational locations. Refill the freezing container periodically with liquid nitrogen to ensure that the cryovials are submerged properly. Place three aluminum strips for subsequent thawing and determination of survival and plant recovery rates together in a single cryovial. As controls for later obtention of the baseline recovery rate, select aluminum strips of the initial, mid, and end phases of the process (e.g., 5th, 10th, and 15th processed strips). Applying this type of sampling, it is easier to identify the potential presence of a cross-contamination event (human error) of the cryovials that are stored in the cryobank.

26. After each accession is processed, place and submerge its cryovial caps in the freezing container containing liquid nitrogen. Cap the cryovials using cotton forceps. Final adjustment of the caps is done by lifting the cryovials with cotton forceps or pressing them down on the freezer holder (to prevent the vials from rotating). The closing of the caps is done with sharp-point pliers, cotton forceps, or hand (protected with cryogenic gloves).
27. This kind of cryotank contains cylindrical canisters in which cryovial holders (aluminum canes) are placed. Using a permanent marker, identify the required number of aluminum canes with the accession identifiers. The cryovial containing the control samples (30 shoot tips) will be placed in a separate canister.
28. First, firmly adjust the cryovial support sections of the aluminum cane. This prevents cryovials from falling into the tank's container when the canes are manipulated. Place the empty canes in one of the containers for precooling them in liquid nitrogen. Let the canes cool down in liquid nitrogen for about 2–3 min. Remove an empty precooled cane from the tank and support it on one of the openings of the freezing container rack. Using sharp-point pliers or cotton forceps, quickly transfer cryovials from the freezing container to the cane. Expose cryovials as little as possible to normal environmental conditions. When a cane has completely been filled with 4–5 vials, immerse the cane again in the corresponding container. Transfer the control vial to the aluminum cane of a separate canister.
29. Label cryoboxes for transitory/cryobank/safety backup storage at its four sides with permanent marker. Additionally, place cryogenic barcoded labels onto the handwritten identifiers. Empty cryoboxes that recirculate from the transitory storage tank; they do not require to be relabeled. Sterilize liquid nitrogen pan and cryoboxes with ultraviolet (UV) light in the LFC for 15 min. Sterilize lids and bases of the cryoboxes separately, with their internal parts facing up (close LFC with UV light-protective panel and leave laboratory during sterilization).
30. Periodically pour additional liquid nitrogen into the pan. The liquid nitrogen should be at such a level that the basal part of the cryovials (0.5–1.0 cm) is submerged in liquid nitrogen, without the cryovials starting to float.
31. The transitory storage cryoboxes have a predefined and fixed location in the cryotank. At CIP, the transitory storage cryotank has six box holders, with a capacity of eight cryoboxes per holder. Holder one contains the cryoboxes 1–8, holder two the boxes 9–16, holder three the boxes 17–23, and so on. The cryoboxes are located by decreasing box identifier in the holders (i.e., for example cryobox 1 is located at the lowest

position in holder one, close to the bottom of the tank; cryobox 2 at the second lowest; cryobox 3 at the third lowest position; and so on). If the transitory storage tank cannot be moved easily (next to the LFC), it is recommended to use a smaller cryobox-based tank as “vehicle” for moving cryoboxes between LFC and transitory storage tank.

32. Place the canes horizontally in the pan, so that all its cryovials are submerged in liquid nitrogen. Transfer up to nine canes to the pan. In the case of CIP, three canes per accession (12 cryovials/accession) are transferred.
33. Verify that the cryovials of each accession are identified with the same code. Additionally, verify that handwritten and barcode labels match on the cryovials. Place a maximum of five accessions per transitory cryobox (12 cryovials per accession). The cryoboxes have a capacity of 100 cryovials. At CIP, each accession occupies two rows inside the box. The remaining free positions of a row are not occupied by other accessions.
34. Close the lid of the cryobox with tissue culture or cotton forceps. At least two people should work together to transfer the cryoboxes. People must wear cryogenic gloves (industrial version), safety goggles, and face shield during the transfer. The first person lifts the holder of the transitory cryotank the box will be transferred to and removes the locking rod. During transfer, the holders should be maintained slightly inclined ($\sim 10^\circ$) toward the person who is lifting the holder, to avoid accidental falling of the cryoboxes. The second person lifts the cryobox on one of its sides with a laboratory spatula, grabs the cryobox on the raised side, and inserts it in the corresponding holder position of the tank. The first person relocates the locking rod of the holder, submerges it in the tanks, and locates the holder in its position. During the transfer, the supports should not be exposed to normal environmental conditions for more than 30–45 s. If you note that the time is not enough, and that the holder and cryoboxes would be exposed more than the allowed time, the holders should be re-submerged (after the locking rod has been located). Let the holder cool down for about 2 min in the tank, before restarting the transfer.
35. The control vials of different accessions are thawed and processed by groups to facilitate their operative handling. Place three test tubes per rack row. Each row corresponds to a separate accession. Disinfect rubber bulbs (1 mL) with alcohol (70%). Install rubber bulb in Pasteur pipette and place pipette in a sterile vessel (e.g., 50 mL centrifuge tube).
36. Place labels (1 × 1 cm) on the test tube caps for the tubes containing RS. The labels contain the following information: accession identifier and “RS:”. Place three large sterile filter papers ($\sim 25 \times 30$ mm) equidistantly in a sterile standard plastic

Petri dish with sterile recovery medium. The filter papers will support the shoot tips during the first stage of the recovery process (first 9 days).

37. Using tissue culture forceps (23 cm), quickly transfer the three aluminum strips of the first accession to be thawed from the control cryovial to the corresponding RS test tubes (placing one strip per test tube). The immersion of the aluminum strips into RS should be quick and immediate. Be careful that all shoot tips are submerged in RS and avoid that they adhere to the test tube wall. If one or more shoot tips adhere to the tube's wall, shake the tube slightly to release the shoot tip(s) with RS solution. Using a marker and temporary label, record the end time of the RS treatment on the tube cap. Monitor RS treatment time with a digital timer. After every 3–5 accessions processed, replace RS pipette with a new sterile one (to reduce the risk of potential cross contamination).
38. The “filter paper sandwich” absorbs the excess RS solution after thawing.
39. To transfer the shoot tips from the small filter papers to recovery medium, slide the small filter paper, with the shoot tips facing down, over one of the large filter papers that is supported by the recovery medium, such that the shoot tips are unloaded. Use the small filter paper to spread the shoot tips evenly over the large filter paper. Verify that the RS test tube cap and the Petri dish with recovery medium are identified with the same accession information. As an additional quality control, place the cryovial label on the lid of the Petri dish with recovery medium the shoot tips will be transferred to for recovery. Let the label dry first before placing it on the Petri dish lid.
40. Place a maximum of five plants per test tube. Rooted samples are transferred directly (= transplant). If the sample has an elongated stem without roots, slightly cut the basal part of the plant to stimulate root formation. Small shoot tips, without stem elongation, are transferred directly. Seal test tubes with one single layer of plastic wrap to facilitate gas exchange or do not seal the tube at all (to have better growth of plants). Sealing of tissue culture vessels contributes to preventing mite infection and associated fungal contamination of the culture.
41. The survived/recovered plants of each Petri dish (30–35-day assessment) are transferred to test tubes, placing a maximum of five plants per tube. The test tubes the plants are transferred to are additionally identified with the original Petri dish ID they were coming from (“1,” “2,” or “3”). Shoot tips that were classified as dead in the first assessment are not transferred to test tubes. Petri dishes that contained 1–5 or 6–10 survived/recovered plants on the 30–35-day assessment will have one or

two test tubes, respectively, for the final assessment (60–65 days after thawing). Recovery assessment of accessions is performed by groups. As accessions are thawed by groups of 40–50 on the same working day, the assessment of its survival/recovery rates will be done by groups as well, 30–35 and 60–65 days after thawing.

42. On some (exceptional) occasions, accessions may show signs of fungal/bacterial contamination during the recovery assessment. Thus, it is required to determine if the source of contamination is a consequence of human error during thawing or media change (and the stored samples in liquid nitrogen are clean without any contamination), or if all cryovials were contaminated during shoot-tip excision, LS/PVS2 treatment, or freezing (and all stored cryovials of the accession are contaminated). Thaw and recover additional samples of the accession. The quantity of retested samples depends on how many petri dishes of the accession (30–35-day assessment) showed signs of contamination, i.e., retest one, two, or three cryovials. If any of the retested cryovials showed signs of contamination, discard the accession from the transitory tank. However, if the retested samples are clean, without any signs of contamination, consider that the accession can be transferred to the cryobank.
43. It is recommended to eliminate any suboptimal cryopreserved accessions (low viability or contamination) before they are transferred to long-term storage. Therefore, at CIP, accessions are eliminated from the transitory tank. Uncap cryovials before discarding to avoid the creation of pressure inside the cryovials. When nitrogen changes its state from liquid to gas, pressure builds up within the vial (volume expansion). Cryovials can explode if they are not uncapped before discarding and cause serious injuries.
44. To reduce risks, cryovials of each accession are conserved for final storage in two separate tanks, the cryobank tank and the safety backup tank.
45. The distribution of cryovials between the cryobank and safety backup boxes varies depending on the recovery rate of the cryo-run. If the cryo-run of an accession showed a recovery rate equal to or greater than 30%, ten cryovials are transferred to the cryobank and two vials to the safety backup box. If the recovery rate is equal to or greater than 20%, but less than 30%, eight vials are transferred to the cryobank, and four vials to the safety backup box. Accessions that show less than 20% of recovery are discarded and not transferred to the cryobank/safety backup boxes. These accessions may require the development of a more specific cryopreservation protocol.
46. It is very useful to have a small cryobox-based laboratory tank available for transporting cryoboxes from the laboratory to the

cryobank. At CIP, a 20-box capacity tank for this purpose is employed. The cryobank and safety backup tanks are organized in a similar way as the transitory tank. They have 12 holders, with a capacity of 15 boxes per holder. The cryoboxes have a predefined position within the holders; that is, holder one contains the cryoboxes 1–15, holder two the boxes 16–30, holder three the boxes 31–45, and so on. The cryoboxes are located by decreasing box identifier in the holders, homologous as for the transitory cryotanks (*see* **Note 31**). Setting up a safety backup copy of the cryo-collection follows the philosophy of not having “all eggs in the same basket.” In case an accession gets lost in the cryobank tank (disasters, human mistake, or other threats), it can be recovered from the safety backup tank. CIP ships its safety backup cryo-collection to a remote place for further reduction of potential risks such as losing the collection and the investment of putting all the potatoes into cryopreservation.

47. Three people work together to transfer the cryoboxes. People should wear cryogenic gloves, safety goggles, and a face shield. During transfer, source (laboratory tank) and destination holders (cryobank or safety backup tanks) should be in the opposite position to allow easy and safe transfer. While one person lifts the holder of the laboratory tank, another person lifts the holder of cryobank/safety backup tank to which the box will be transferred and removes the locking rod. During transfer, the holders should be maintained slightly inclined ($\sim 10^\circ$) toward the person who is lifting the holder, to avoid accidental falling of cryoboxes. The third person transfers the cryobox from the laboratory tank to the corresponding position of the cryobank/safety backup tank. The cryobox holders have a hole in their back that allows the box to be pushed lightly with forceps and then held securely with the other free hand. Relocate the locking rods of the holders, submerge the holders in the tanks, and place them in their corresponding position. If several boxes have the same origin and destination holder, up to three boxes can be transferred successively, without submerging the holders after each transferred box. During the transfer, the supports should not be exposed to normal environmental conditions for more than 30–45 s. If you note that the time is not enough, and that the holders and cryoboxes would be exposed more than the allowed time, the holders should be re-submerged (after the locking rod has been located). Allow the supports to cool for about 2 min in the tanks, before restarting the transfer.
48. Each type of tank has a specific minimum liquid nitrogen level, depending on the tank's net evaporation rate, internal architecture, isolation, etc. Cryotanks that store samples are

low-pressure tanks; that is, liquid nitrogen continuously evaporates and must be replaced. The cryotanks always should be refilled before the minimum liquid nitrogen level is reached. If the liquid nitrogen level of the tank decreases below this minimum, the temperature within the tank increases to a suboptimum value (especially in the uppermost part of the tank, far away from the surface of liquid nitrogen), and the viability of the samples is at risk. It is recommended to equip the cryobank and safety backup tanks with liquid nitrogen-monitoring devices that electronically and automatically record the liquid nitrogen level of the tanks (probes at two points), internal tank temperature, and other variables (e.g., consumption of liquid nitrogen). Additionally, record the tank's liquid nitrogen level and temperature every day manually (taking notes of the values shown on the monitor display). Liquid nitrogen probes should be calibrated periodically, according to a pre-established preventive maintenance plan. In the case of smaller cryotanks, e.g., laboratory tanks, the liquid nitrogen level is measured weekly using a cryogenic measuring rod.

49. Filling of tanks can be done with completely automatized systems or semi-automatized, depending on the extension of the cryobank and volume of liquid nitrogen available. In a completely automatized system tanks are connected to a high-pressure liquid nitrogen tank of large capacity, and filling is triggered automatically by computer-controlled valves, according to a predefined schedule. Filling time and/or volume of each tank is controlled individually. In smaller scale cryobanks, filling of liquid nitrogen tanks is done semiautomatically, connecting each individual tank, subsequently, with liquid nitrogen transfer hose to a medium-sized high-pressure liquid nitrogen tank. Starting and stopping of the filling process are triggered manually. Laboratory tanks and Dewars, that are not equipped with filling disposals, are filled manually (i.e., a person holds the liquid nitrogen transfer hose during filling).

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