Cryopreservation of tobacco BY-2 suspension cell cultures[1]

Version 1.3 (2022.9.12)

[1] This protocol was translated from the Japanese version that had been used in RIKEN BRC Technical Training Course.

Change history

Version 1.0 (2018.11.30)

- Translated from Japanese version.

Version 1.1 (2019.9.3)

- Added subsection 1.1 Plant cell culture in section 1 Materials.
- modified Appendix A.
- Updated link url.

Version 1.2 (2022.8.1)

- Changed E-mail address plant@brc.riken.jp to plant.brc@riken.jp.

Version 1.3 (2022.9.12)

- Corrected footnotes.
- Replaced Wako Pure Chemical Industries with FUJIFILM Wako Pure Chemical Corporation and TOMY Seiko with TOMY Digital Biology Co., Ltd.
- Added equipment to 1.3 Equipment section and sorted the list.
- Replaced 25°C with room temperature in 2.1 Cryopreservation 13.
- Deleted Appendix A “Cryopreservation of Arabidopsis T87 suspension cell cultures.”
- Corrected typographical errors and wordings.

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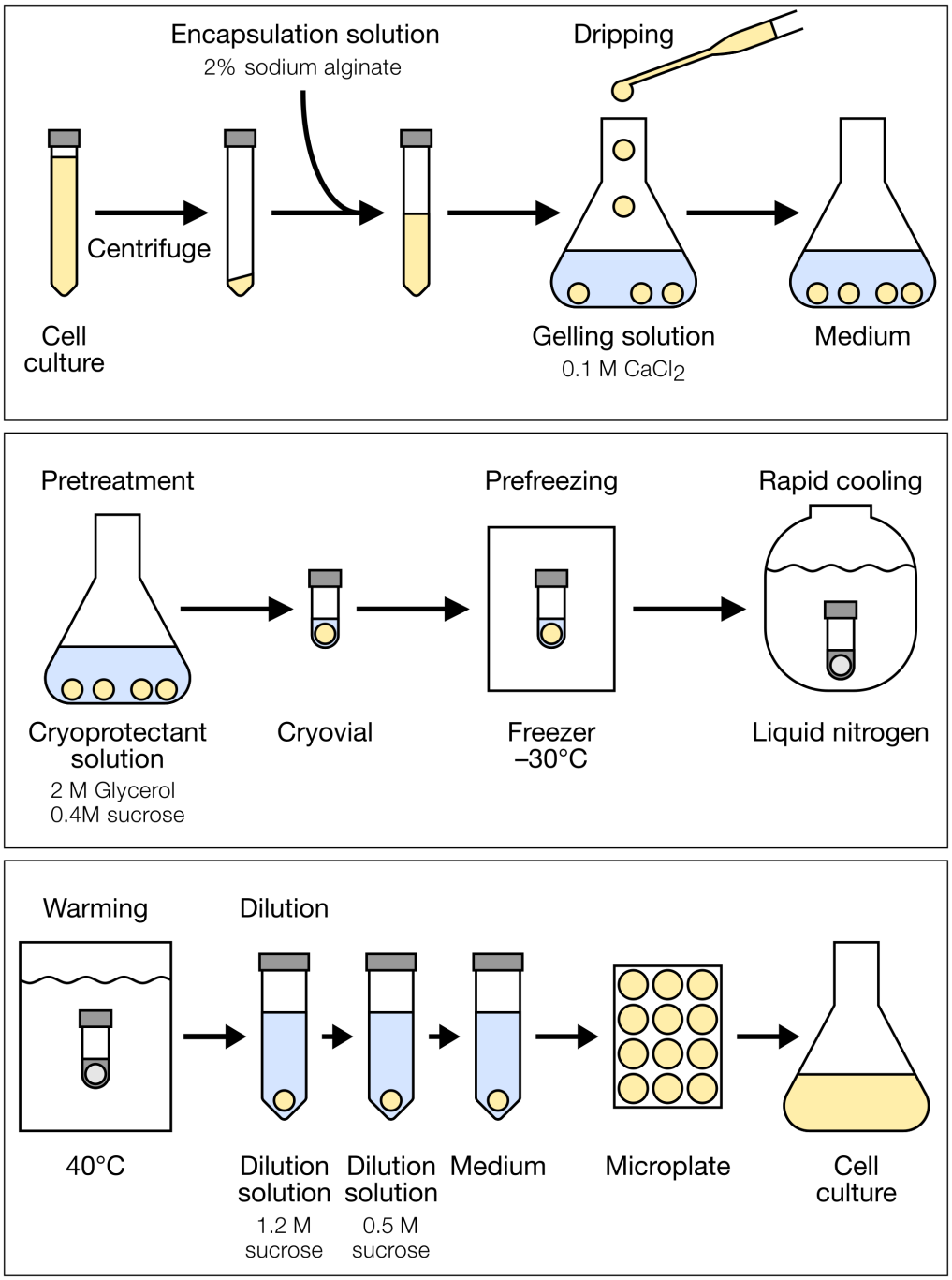

Figure 1: Schematic diagram of cryopreservation procedure

1 Materials

1.1 Plant cell culture

- Tobacco BY-2 suspension cell cultures[2], after 3 days of subculturing[3]

1.2 Chemicals

■ Cryopreservation

A) Culture medium: modified Linsmaier and Skoog (mLS) medium, 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.8[4]

MS Plant Salt Mixture	1 bag
Sucrose	30 g
BY2_P	2.5 mL
LS_VT_modified	2.5 mL
2,4-D (0.2 mg/mL)	1 mL
	1 L

Adjust pH to 5.8, sterilize by autoclave.

B) Encapsulation solution: medium containing 2% (w/v) sodium alginate

Culture medium[5]	100 mL
Sodium alginate[6]	2 g

Dissolve by stirring under heating at about 60°C.
Sterilize by autoclave.

C) 3 M $CaCl_2$ solution

$CaCl_2 \cdot H_2O$	22.1 g
	50 mL

Sterilize by filtration or autoclave.

[2] RIKEN BRC plant cell line documentation (rpc00001; https://plant.rtc.riken.jp/resource/cell_line/web_documents/cell_lines/rpc00001.html)

[3] Cultured cells are taken from the exponential growth phase. The cells are small and have rich cytoplasm with small vacuoles.

[4] RIKEN BRC plant cell line documentation (medium no. 1; https://plant.rtc.riken.jp/resource/cell_line/web_documents/media/medium_1.html)

[5] Sodium alginate is usually dissolve in calcium-free medium. We were able to dissolve sodium alginate in common mLS medium, because the calcium chloride concentration of the medium (3 mM) does not induce gelation of alginate.

[6] Sodium alginate 300–400 (No. 190-09991, FUJIFILM Wako Pure Chemical Corporation)

D) Gelling solution: medium containing 0.1 M $CaCl_2$

Culture medium, sterilized	60 mL
3 M $CaCl_2$ solution, sterilized	2 mL

E) 2× Medium: double-strength mLS medium, not containing sucrose

Murashige and Skoog Salt Mixture	1 bag
BY2_P	2.5 mL
LS_VT_modified	2.5 mL
2,4-D (0.2 mg/mL)	1 mL
	500 mL

F) Cryoprotectant solution: medium containing 2 M glycerol and 0.4 M sucrose

2× Medium	150 mL
Glycerol	55.3 g
Sucrose	41.1 g
	300 mL

Adjust pH to 5.8, sterilize by autoclave.

■ Regrowth

G) Dilution solution (1.2 M): medium containing 1.2 M sucrose

2× Medium	150 mL
Sucrose	123.2 g
	300 mL

Adjust pH to 5.8, sterilize by autoclave.

H) Dilution solution (0.5 M): medium containing 0.5 M sucrose

2× Medium	150 mL
Sucrose	51.3 g
	300 mL

Adjust pH to 5.8, sterilize by autoclave.

■ Evaluation of cell viability

I) 10 mg mL^{-1} Evans blue solution

Evans blue	100 mg

10 mL

J) Staining solution: medium containing 1 mg mL^{-1} Evans blue

Culture medium	9 mL
10 mg mL^{-1} Evans blue solution	1 mL

1.3 Equipment

■ **Cryopreservation**

- Microscope
- Conical tube, 15 mL
- Low-speed centrifuge
- Pipette
- Erlenmeyer flask, 200 mL
- Pasteur pipette
- Shaker
- Cryovial, 2.0 mL, round bottom[7]
- Forceps
- Vial rack[8] (Figure 2)
- Laboratory freezer, −30°C
- Cane for cryovials
- Dewar flask

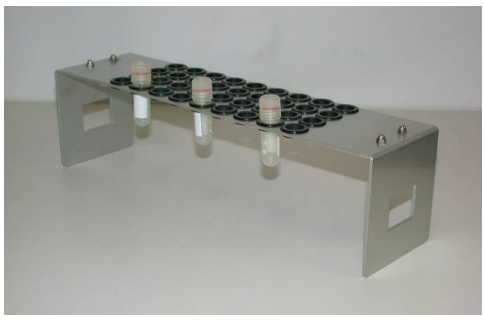

Figure 2: 1.5 (2) mL tube rack TR-4002 (Micro tube mixer MT-400 supplied rack; TOMY Digital Biology Co., Ltd.)

■ **Regrowth**

- Conical tube, 50 mL
- Water bath
- Shaker
- Pipette
- Forceps
- Cell culture plate, 12 well[9]
- Micro spatula

[7] Cryo.s™, 2 mL, PP, round bottom, internal thread (Item No. 121263, Greiner Bio-One)

[6] Do not use a rack that cover the bottom of the cryovials.

[9] Falcon® 12 well clear flat bottom not treated multiwell cell culture plate (product #351143, Corning)

■ **Evaluation of cell viability**

- Surgical blade
- Pipette
- Cell culture plate, 12 well
- Forceps
- Microscope slide
- Cover slip
- Microscope

2 Methods

2.1 Cryopreservation

1. Check physiological condition of cultured cells by observing them under a microscope.[10]
2. Transfer suspension cell culture into a 15-mL conical tube.
3. Centrifuge the tube at 100 $\times g$ for 5 min.
4. Check volume of the pelleted cells and remove the supernatant with a pipette.
5. Gently suspend the pelleted cells in 3–4 volume of encapsulation solution.
6. Pour 60 mL of gelling solution to a 200-mL Erlenmeyer flask.
7. Drip the mixture of cells and encapsulation solution into the gelling solution with a Pasteur pipette[11].[12]
8. Keep the beads formed from the encapsulated cells in the gelling solution with gentle shaking for 5–10 min.
9. Remove the gelling solution with a pipette.
10. Wash the beads with 10 mL of culture medium: Add culture medium, gently swirl the Erlenmeyer flask, and remove the culture medium with a pipette.
11. Incubate the beads in 50 mL of culture medium for 10–20 min.
12. Remove the culture medium and wash the beads with 10 mL of cryoprotectant solution.

[10] Good physiological condition of the cultured cells is essential for successful cryopreservation.

[11] Either Gilson PIPETMAN P-1000 or disposable 2-mL pipette can be used instead of a Pasteur pipette.

[12] The alginate gel beads about 4 mm in diameter (about 30 µL) are formed immediately after dripping.

13. Incubate the beads in 50 mL[13] of cryoprotectant solution at room temperature for 60 min with gentle shaking (pretreatment[14]).
14. Pour 300 µL of the cryoprotectant solution to a 2-mL cryovial.
15. Transfer three beads into each cryovial with forceps.[15]
16. Place the cryovials in a rack and store them in a laboratory freezer at −30°C for 2 h (slow prefreezing[16]).[17]
17. After removing the cryovials from the freezer, immediately set the cryovials to cryovial canes and immerse it in liquid nitrogen (rapid cooling[18]).
18. Store the cryovials in vapor phase of a liquid nitrogen storage tank.[19]

2.2 Regrowth

1. Pour 30 mL of dilution solution (1.2 M) to a 50-mL conical tube.
2. Warm each cryovial in a water bath at 40°C with gentle agitation.[20]
3. After thawing, immediately remove the cryovials from the bath.
4. Transfer the three beads and cryoprotectant solution in the conical tube containing dilution solution (1.2 M).[21]
5. Set the conical tube horizontally on a shaker and incubate the beads at room temperature for 15 min with gentle shaking.
6. Replace the dilution solution (1.2 M) with 30 mL of dilution solution (0.5 M): Remove the dilution solution (1.2 M) with a pipette and add dilution solution (0.5 M) to the conical tube.
7. Incubate the beads for 15 min with gentle shaking.

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[13] The beads are suspended in at least 1 mL of cryoprotectant solution per bead.

[14] The cryoprotectant pretreatment promotes tolerance of cells to cooling to −30°C and subsequent exposure to liquid nitrogen.

[15] Total volume of the sample is about 400 µL.

[16] The slow prefreezing causes freeze-induced dehydration of cells.

[17] The slow prefreezing can be achieved with simple cooling in a laboratory freezer rather than with controlled-rate cooling in a programmable freezer. The cooling rate may be affected by some environmental factors, e.g., sample volume, cooling position in a freezer, and space between the cryovials.

[18] The dehydrated cells are vitrified by rapid cooling in liquid nitrogen. The vitrified cells can be preserved safely at the temperature of liquid nitrogen (−196°C) for an indefinite length of time.

[19] The viability of cells is checked using one cryovial before long-term storage.

[20] In order to avoid recrystallization of the vitrified cells, it is necessary to warm the cryovial rapidly. Also it is important not to overheat.

[21] The cryoprotectant solution is stepwisely diluted to prevent the damage caused by rapid change in osmotic pressure.

8. Replace the dilution solution (0.5 M) with 30 mL of culture medium and incubate the beads for 15 min with gentle shaking.
9. Suspend three beads in 3 mL of fresh culture medium in each well of a 12-well cell culture plate.
10. Culture the beads at 27°C in the dark for 3 days with shaking at 130 rpm.
11. Gently crush the beads with a micro spatula to release the encapsulated cells into the culture medium.[22]
12. Culture the cell suspension for an additional 4 days.
13. Transfer the cell suspension to 95 mL of fresh culture medium in a 300-mL Erlenmeyer flask.

2.3 Evaluation of cell viability

1. Cut the bead into two to four pieces.[23]
2. Soak the pieces in 1 mL of Evans blue staining solution in each well of a 12-well cell culture plate for 20 min.
3. Transfer the pieces to 1 mL of culture medium and incubate them for 20 min.
4. Place one piece of the bead on a microscope slide and gently crush with a cover slip.
5. Count living and died cells, respectively.[24]

[22] The beads must be cultured until the embedded cells proliferate vigorously.

[23] The cell viability is determined after 1 day of culture, because we could not evaluate the viability of cells that were still recovering from cryopreservation immediately after re-warming.

[24] Died cells are stained blue.

3 References

- Kobayashi T, Niino T, Kobayashi M (2005) Simple cryopreservation protocol with an encapsulation technique for tobacco BY-2 suspension cell cultures. Plant Biotechnology 22: 105–112. DOI: 10.5511/plantbiotechnology.22.105
- Kobayashi T, Niino T, Kobayashi M (2006) Cryopreservation of tobacco BY-2 suspension cell cultures by vitrification with encapsulation. Plant Biotechnology 23: 333–338. DOI: 10.5511/plantbiotechnology.23.333
- Menges M, Murray JAH (2004) Cryopreservation of transformed and wild-type Arabidopsis and tobacco cell suspension cultures. Plant Journal 37: 635–644. DOI: 10.1046/j.1365-313X.2003.01980.x
- Ogawa Y, Sakurai N, Oikawa A, Kai K, Morishita Y, Mori K, Moriya K, Fujii F, Aoki K, Suzuki H, Ohta D, Saito K, Shibata D (2012) High-throughput cryopreservation of plant cell cultures for functional genomics. Plant and Cell Physiology 53: 943–952. DOI: 10.1093/pcp/pcs038
- Sakai A, Kobayashi S, Oiyama I (1991) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb.) by simple freezing method. Plant Science 74: 243–248. DOI: 10.1016/0168-9452(91)90052-A
- Sakai A (1996) Cryopreservation of cultured plant cells and meristems. Plant tissue culture letters 13: 1–6. (in Japanese) 10.5511/plantbiotechnology1984.13.1
- Hirai D (2001) Studies on cryopreservation of vegetatively propagated crops by encapsulation-vitrification method. Report of Hokkaido prefectural agricultural experiment stations No. 99. (in Japanese) https://agriknowledge.affrc.go.jp/RN/2010630232.pdf