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

Version 1.2 (2022.8.1)

[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.

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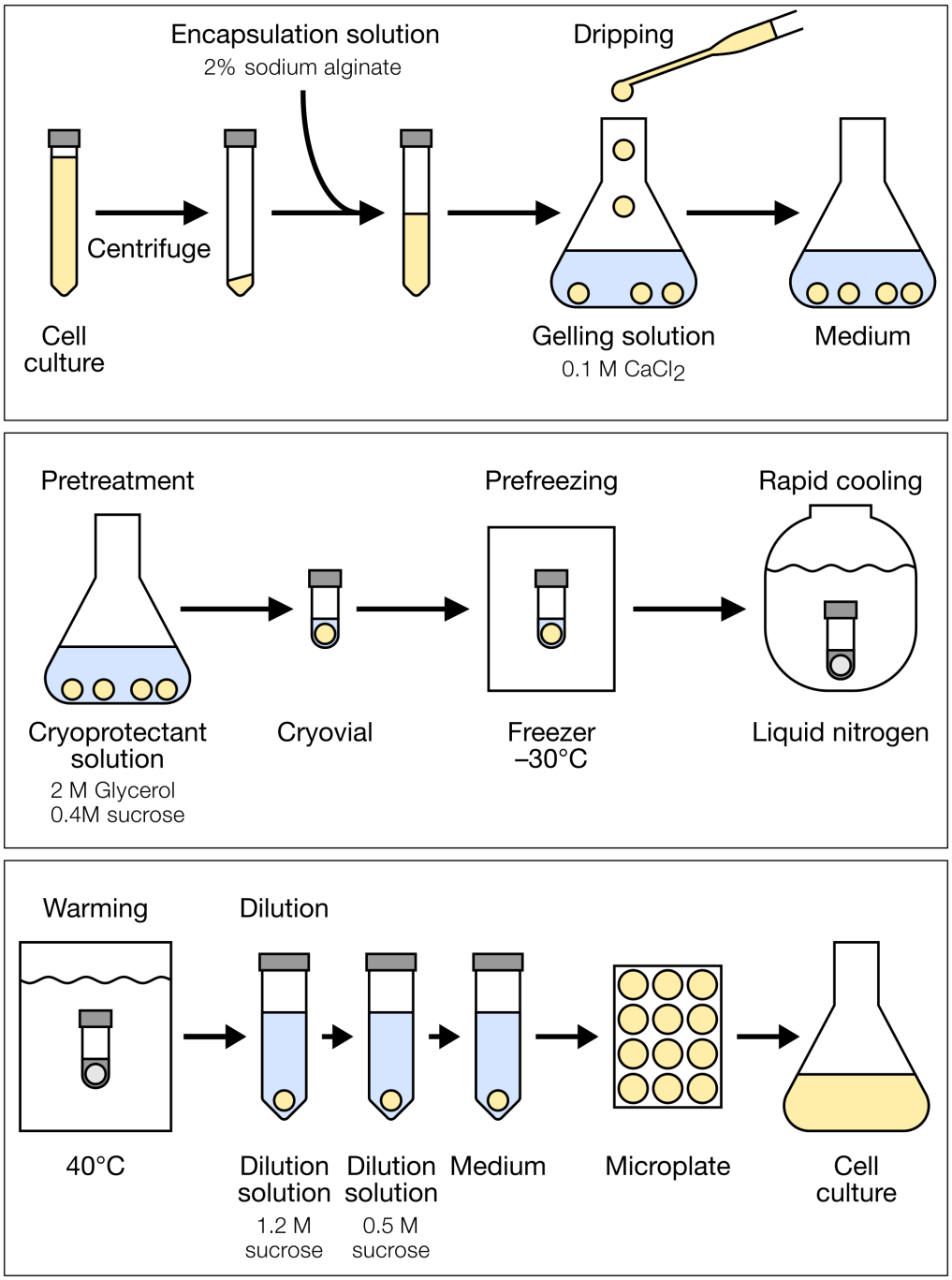

Figure 1: Schematic diagram of cryopreservation procedure

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[5]

Culture medium	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)

[3]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.

[4]Sodium alginate 300–400 (No. 190-09991, Wako Pure Chemical Industries)

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**

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

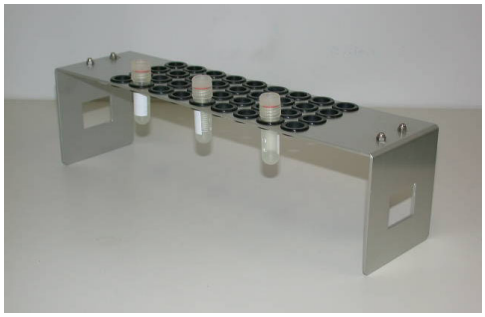

Figure 2: 1.5 (2) mL tube rack TR-4002 (Micro tube mixer MT-400 supplied rack; TOMY Seiko)

■ **Regrowth**

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

[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
- 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 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 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 with gentle shaking at 25°C for 60 min (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 with gentle shaking for 15 min at room temperature.
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 with gentle shaking for 15 min.

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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 with gentle shaking for 15 min.
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 with shaking at 130 rpm in the dark for 3 days at 27°C.
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 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

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A Cryopreservation of Arabidopsis T87 suspension cell cultures: Differences from that of tobacco BY-2 suspension cell cultures

A.1 Materials

- Arabidopsis T87 suspension cell cultures[25], after 5 days of subculturing[26]
- Jouanneau and Péaud-Lenoël (JPL) medium[27]
- Solutions prepared from JPL medium instead of mLS medium
- Stainless sieve (diameter, 5 cm; pore size, 300 µm) set on a tall beaker (200 mL)

A.2 Methods

1. Encapsulation: Select small clusters of T87 cells by passing T87 cell suspension through a 300-µm sieve. After centrifugation, suspend the pelleted cells in 1–2 volume of encapsulation solution.
2. Cryopreservation: Incubate beads encapsulating T87 cells in cryoprotectant solution for 40 min, store them in a freezer at -30°C for 3 h, and then rapidly cool them in liquid nitrogen.
3. Regrowth: After dilution of cryoprotectant solution, culture the beads in JPL medium for 3 days. Crushed the beads with a micro spatula, and culture the cell suspension for an additional 10–14 days.

[25] RIKEN BRC plant cell line documentation (rpc00008; https://plant.rtc.riken.jp/resource/cell_line/web_documents/cell_lines/rpc00008.html)

[26] Cultured cells are taken from the exponential growth phase.

[27] RIKEN BRC plant cell line documentation (medium no. 5; https://plant.rtc.riken.jp/resource/cell_line/web_documents/media/medium_5.html)