Cryopreservation of Arabidopsis T87 suspension cell cultures

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Change history

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1 Materials

1.1 Plant cell culture

- *Arabidopsis thaliana* T87 suspension cell culture[1], after 5 days of subculturing[2]

1.2 Chemicals

■ Cryopreservation

A) Culture medium: Jouanneau and Péaud-Lenoël (JPL) medium, 1 µM 1-naphthaleneacetic acid (NAA)[3]

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

Culture medium[4]	100 mL
Sodium alginate[5]	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.

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

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

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

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

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

[4] Sodium alginate is usually dissolve in calcium-free medium. We were able to dissolve sodium alginate in common JPL medium, because the calcium chloride concentration of the medium (0.9 mM) does not induce gelation of alginate.

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

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

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 5.7, 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 5.7, 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 5.7, 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

K) 0.2 M K-phosphate buffer, pH 7.5

KH_2PO_4	2.18 g
K_2HPO_4	14.62 g
	500 mL

L) 50 mM K-phosphate buffer

0.2 M K-phosphate buffer	50 mL
	200 mL

M) 0.6% (w/v) 2,3,5-Triphenyl tetrazorium chloride (TTC) solution

TTC	600 mg
0.2 M K-phosphate buffer	25 mL
	100 mL

N) Ethanol, 99.5%

1.3 Equipment

■ Cryopreservation

- Microscope
- Tall beaker, 200 mL
- Stainless sieve, diameter 5 cm, pore size 300 µm; set on a tall beaker
- Conical tube, 15 mL
- Low-speed centrifuge
- Pipette
- Erlenmeyer flask, 200 mL
- Pasteur pipette
- Shaker
- Cryovial, 2.0 mL, round bottom[6]
- Forceps

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

- Vial rack[7] (Figure 1)
- Laboratory freezer, −30°C
- Cane for cryovials
- Dewar flask

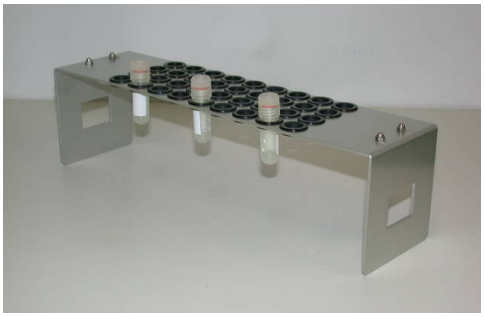

Figure 1: 1.5 (2) mL tube rack TR-4002 (Micro tube mixer MT-400 supplied rack; TOMY Digital Biology Co., Ltd.)

■ **Regrowth**

- Conical tube, 50 mL
- Water bath
- Shaker
- Pipette
- Forceps
- Cell culture plate, 12 well[8]
- Micro spatula

■ **Evaluation of cell viability**

- Surgical blade
- Pipette
- Cell culture plate, 12 well
- Forceps
- Microscope slide
- Cover slip
- Microscope
- Conical tube, 15 mL
- Low-speed centrifuge
- Water bath
- Micro spatula
- Spectrophotometer

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

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

2 Methods

2.1 Cryopreservation

1. Check physiological condition of cultured cells by observing them under a microscope.[9]
2. Pass suspension cell culture through a 300-µm sieve.
3. Transfer suspension cell culture into a 15-mL conical tube.
4. Centrifuge the tube at 100 $\times g$ for 5 min.
5. Check volume of the pelleted cells and remove the supernatant with a pipette.
6. Gently suspend the pelleted cells in 1–2 volume of encapsulation solution.
7. Pour 60 mL of gelling solution to a 200-mL Erlenmeyer flask.
8. Drip the mixture of cells and encapsulation solution into the gelling solution with a Pasteur pipette[10].[11]
9. Keep the beads formed from the encapsulated cells in the gelling solution with gentle shaking for 5–10 min.
10. Remove the gelling solution with a pipette.
11. 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.
12. Incubate the beads in 50 mL of culture medium for 10–20 min.
13. Remove the culture medium and wash the beads with 10 mL of cryoprotectant solution.
14. Incubate the beads in 50 mL[12] of cryoprotectant solution at room temperature for 40 min with gentle shaking (pretreatment[13]).
15. Pour 300 µL of the cryoprotectant solution to a 2-mL cryovial.
16. Transfer three beads into each cryovial with forceps.[14]

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

[10] A disposable 2-mL pipette can be used instead of a Pasteur pipette.

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

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

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

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

17. Place the cryovials in a rack and store them in a laboratory freezer at −30°C for 3 h (slow prefreezing[15]).[16]

18. After removing the cryovials from the freezer, immediately set the cryovials to cryovial canes and immerse it in liquid nitrogen (rapid cooling[17]).

19. Store the cryovials in vapor phase of a liquid nitrogen storage tank.[18]

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.[19]

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).[20]

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.

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 22°C under the continuous light for 3 days with shaking at 120 rpm.

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

[16] 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.

[17] 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.

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

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

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

11. Gently crush the beads with a micro spatula to release the encapsulated cells into the culture medium.[21]
12. Culture the cell suspension for an additional 10–14 days.
13. Transfer the cell suspension to 80 mL of fresh culture medium in a 300-mL Erlenmeyer flask.

2.3 Evaluation of cell viability

■ Evans blue staining

1. Cut the bead into two to four pieces.[22]
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. Observe the cultured cells under a microscope.[23]

■ TTC assay

1. Transfer 3–5 beads to a 15-mL conical tube containing 3 mL of 50 mM K-phosphate buffer.
2. Incubate the conical tube for 10 min.
3. Discard the buffer by a pipette.
4. Add 3 mL of 0.6% (w/v) TTC solution.
5. Incubate the conical tube in darkness at 22°C for 6 h.
6. Centrifuge the conical tube at 3,000 rpm for 1 min.
7. Discard TTC solution by a pipette.
8. Add 3 mL of 50 mM K-phosphate buffer.
9. Incubate the conical tube for 10 min.
10. Centrifuge the conical tube at 3,000 rpm for 1 min.
11. Discard the buffer by a pipette.

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

[22] 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.

[23] Died cells are stained blue.

12. Crash the beads with a spatula.
13. Add ethanol to 3 mL.
14. Incubate the conical tube in a water bath at 60°C for 10 min.
15. Centrifuge the conical tube at 3,000 rpm for 1 min.
16. Measure absorbance at 485 nm using a spectrophotometer.
17. Calculate cell viability relative to the non-treated control.[24]

[24] Prepare the following samples: 1) Non-treated beads (100%). 2) Non-treated frozen beads (0%): The cells are not alive. 3) Cryopreserved beads (Sample). The cell viability can be calculated as: (A_{485}Sample $-$ A_{485}0%) / (A_{485}100% $-$ A_{485}0%) $\times$ 100(%)