Isolation of intact vacuoles from *Catharanthus* suspension-cultured cells

Preparation of protoplasts

In the case of about 24 g cells (Fresh weight from 160 ml suspension-cultured cells)

Remove the medium and wash the cells with the washing solution by aspiration

Solution A

Cellulase Y-C	1 g (1 %)
Pectolyase Y-23	50 mg (0.05 %)
P sor (0.5 M) pH 6.0	100 ml

↓

Incubate the cells in the solution A for 2.7 h at 31℃ with shaking at 100 - 120 rpm

Purification of vacuoles

Move the released protoplasts to the 50 ml tube

Underlayed with Vc suc (0.4 M)

↓

Centrifugation (200×g, 10 min)

↓

Remove the supernatant

↓

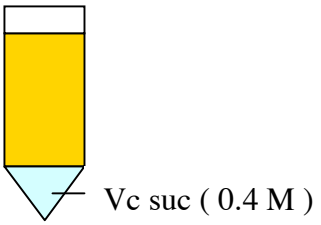
Add Vc suc

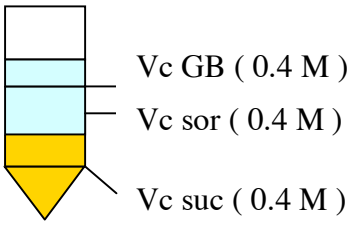
up to 15 ml and mix well

Form a gradient by overlaying

7 ml Vc sor (0.4 M) and

2 ml Vc GB (0.4 M)





↓

Centrifugation (200×g, 1-2 min, and then 1600×g, 8 min)

↓

Remove the solution over the purified protoplasts

Transfer the protoplasts to new tube

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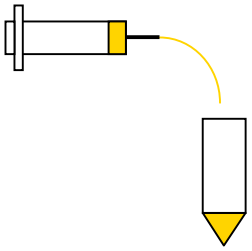
Add equal volume of Vc med 0 and vortex

Incubate on the ice for 5 min

↓

Squeeze through a syringe 19 G (1.10 × 90 mm)



Add equal volume of VcGB and underlayed with Vc suc (0.4 M)

↓

Centrifugation (200×g, 1-2 min, and then 1600×g, 8 min)

↓

Remove the supernatant

↓

Divide the pellet into two glass tubes

↓

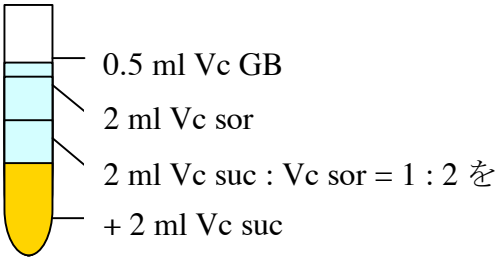
Add Vc suc (0.4 M) to half the tube

Form a gradient by overlaying

2 ml Vc suc : Vc sor = 1 : 2,

2 ml Vc sor, and

0.5 ml Vc GB



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Centrifugation (200×g, 1-2 min, and then 1600×g, 8 min)

↓

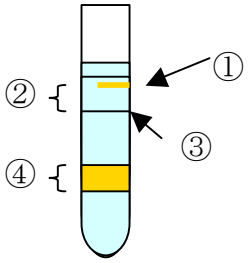
Vacuoles is obtained in the interphase (①)

between Vc GB and Vc sor

Collect solution (②)

After confirm vacuoles in the interphase (③),

Collect vacuoles



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Remove the layers over ④

↓

Squeeze through a syringe 19 G (1.10 × 90 mm) storonger than first time

↓

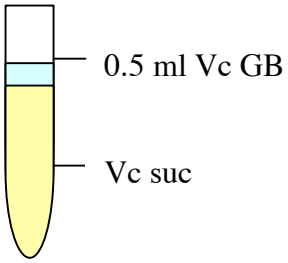
As described above, form a gradient

↓

Centrifugation (1000 rpm, 1-2 min, and then 3000 rpm, 8 min)

↓

Collect vacuoles



↓

In order to concentrate of vacuoles,

Add Vc suc (0.4 M) to

the solution of vacuoles, and overlay

Vc GB (0.4 M)

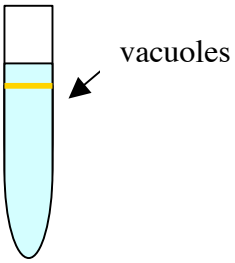
↓

Centrifugation

(1000 rpm, 1-2 min, and then 3000 rpm, 8 min)

↓

Collect vacuoles



Stock solutions

Protoplast 10 x	500 ml
Mes (213.25)	10.6625 g (100 mM)
$CaCl_2 \cdot 2H_2O$ (147.02)	0.7351 g (10 mM)
pH 6.0 (Tris)	

Vc med 10 x	1000 ml
HEPES (238.3)	71.49 g (300 mM)
EGTA (380.4)	7.608 g (20 mM)
Potassium gluconate (234.2)	70.26 g (300 mM)
$MgCl_2$ (203.3)	4.066 g (20 mM)
pH 7.2 with Tris	

Solutions

P sor (0.5 M) pH 6.0

10 mM Mes

1 mM $CaCl_2 \cdot 2H_2O$

0.5 M sorbitol

Washing solution

2 mM $CaSO_4 \cdot 2H_2O$

100 mM Sorbitol

Vc med 0

30 mM HEPES

2 mM EGTA

30 mM Potassium gluconate

2 mM $MgCl_2$

pH 7.2 with Tris

Vc suc (0.4 M)	200 ml
Vc med 10 x	20 ml
2M sucrose	40 ml

Vc sor (0.4 M)	200 ml
Vc med 10 x	20 ml
2M sorbitol	40 ml

Vc GB (0.4 M)	200 ml
Vc med 10 x	20 ml
2M betaine monohydrate	40 ml

Vc suc : Vc sor = 1 : 2