

## Isolation of intact vacuoles from *Arabidopsis* suspension-cultured cells

### Preparation of protoplasts

In the case of about 12 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	1.6 g ( 1 % )
Pectolyase Y-23	160 mg ( 0.1 % )
P sor ( 0.5 M ) pH 6.0	160 ml

↓

Incubate the cells in the solution A for 2.8 - 3.3 h at 31°C with shaking at 110 - 120 rpm

### Purification of vacuoles

Move the released protoplasts to the 50 ml tube

Underlayed with Vc sor ( 0.4 M ) 50 % percoll

↓

Centrifugation ( 200 × g, 10 min )

↓

Remove the supernatant

↓

Add Vc sor ( 0.4 M ) 50 % percoll

up to 15 ml and mix well

Form a gradient by overlaying

10 ml Vc sor ( 0.4 M ) 7.5 % percoll and

2 ml Vc sor ( 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

↓

Add equal volume of Vc med 0 and vortex

Incubate on the ice for 5 min

↓



Vc sor ( 0.4 M ) 50 % percoll



Vc sor ( 0.4 M )

Vc sor ( 0.4 M ) 7.5 % percoll

Vc sor ( 0.4 M ) 50 % percoll

Divide the solution into eight glass tubes



Add 1.5 ml Vc sor ( 0.2 M ) 25 % percoll and mix

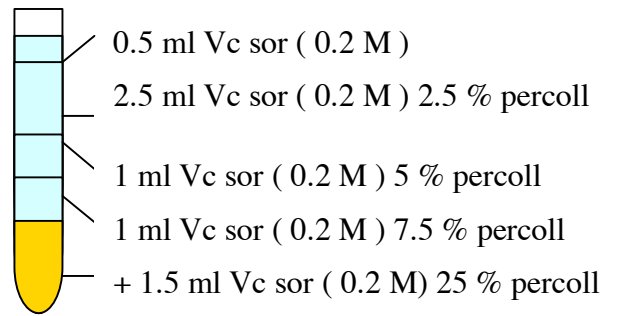
Form a gradient by overlaying

1 ml Vc sor ( 0.2 M ) 7.5 % percoll,

1 ml Vc sor ( 0.2 M ) 5 % percoll,

1.5 ml Vc sor ( 0.2 M ) 2.5 % percoll, and

0.5 ml Vc sor ( 0.2 M )



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



Vacuoles is obtained in the interphase ( ① )

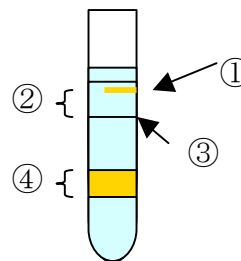
between Vc sor ( 0.2 M )

and Vc sor ( 0.2 M ) 2.5 % percoll

Collect solution ( ② )

After confirm vacuoles in the interphase ( ③ ),

Collect vacuoles



Remove the layers over ④



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



As described above, form a gradient



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



Collect vacuoles

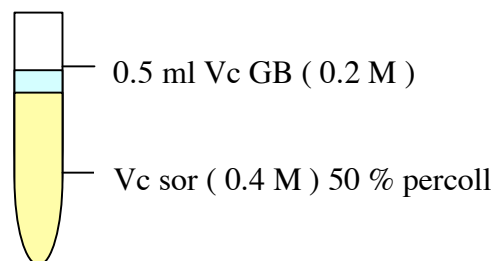


In order to concentrate of vacuoles,

Add Vc sor ( 0.4 M ) 50 % percoll to

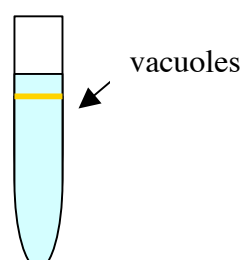
the solution of vacuoles, and overlay

Vc GB ( 0.2 M )



Centrifugation

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



Collect vacuoles

↓

In case of removal percoll,

Form a gradient by overlaying

Vc sor ( 0.4 M ) 50 % percoll

Vc suc ( 0.4 M )

Vc GB ( 0.2 M )

↓

Centrifugation (  $200 \times g$ , 1-2 min, and then  $1600 \times g$ , 8 min )

↓

Collect vacuoles

## Stock solutions

Protoplast 10 x                    500 ml  
Mes ( 213.25 )                    10.6625 g ( 100 mM )  
CaCl<sub>2</sub> · 2H<sub>2</sub>O ( 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<sub>2</sub> ( 203.3 )                    4.066 g ( 20 mM )  
pH 7.2 with Tris

## Solutions

### Washing solution

2 mM CaSO<sub>4</sub> · 2H<sub>2</sub>O  
100 mM Sorbitol

### P sor ( 0.5 M ) pH 6.0

10 mM Mes  
1 mM CaCl<sub>2</sub> · 2H<sub>2</sub>O  
0.5 M sorbitol  
pH 6.0 with Tris

### Vc med 0

30 mM HEPES  
2 mM EGTA  
30 mM Potassium gluconate  
2 mM MgCl<sub>2</sub>  
pH 7.2 with Tris

Vc sor ( 0.4 M ) 50 % percoll                    200 ml  
Vc med 10 x                    20 ml  
2M sorbitol                    40 ml

percoll	100 ml
H <sub>2</sub> O	40 ml

Vc sor ( 0.4 M ) 7.5 % percoll	200 ml
Vc med 10 x	20 ml
2M sorbitol	40 ml
percoll	15 ml
H <sub>2</sub> O	125 ml

Vc sor ( 0.2 M ) 25 % percoll	200 ml
Vc med 10 x	20 ml
2M sorbitol	20 ml
percoll	50 ml
H <sub>2</sub> O	110 ml

Vc sor ( 0.2 M ) 7.5 % percoll	200 ml
Vc med 10 x	20 ml
2M sorbitol	20 ml
percoll	15 ml
H <sub>2</sub> O	145 ml

Vc sor ( 0.2 M ) 5 % percoll	200 ml
Vc med 10 x	20 ml
2M sorbitol	20 ml
percoll	10 ml
H <sub>2</sub> O	150 ml

Vc sor ( 0.2 M ) 2.5 % percoll	200 ml
Vc med 10 x	20 ml
2M sorbitol	20 ml
percoll	5 ml
H <sub>2</sub> O	155 ml

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

H<sub>2</sub>O 140 ml

Vc sor ( 0.4 M ) 200 ml

Vc med 10 x 20 ml

2M sorbitol 40 ml

H<sub>2</sub>O 140 ml

Vc sor ( 0.2 M ) 200 ml

Vc med 10 x 20 ml

2M sorbitol 20 ml

H<sub>2</sub>O 160 ml

Vc GB ( 0.2 M ) 200 ml

Vc med 10 x 20 ml

2M betaine monohydrate 20 ml

H<sub>2</sub>O 160 ml