

## **PROTOCOLS & RECIPES**

### **Protoplast Preparation**

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#### **Protocol for the preparation of metabolically competent protoplasts**

Method for isolating large numbers of metabolically competent protoplasts from leaves of monocotyledons (grasses), dicotyledons (such as spinach and sunflower) (Ref. 1 - 3) or from hypocotyl tissue (e.g. *Brassica napus*) (Ref. 4).

- (1)** Leaf slices of monocots and dicots are prepared by cutting the leaves with a sharp razor blade into segments 0.5 - 1 mm in size. In the case of dicots the epidermis can be scraped off before cutting by rubbing with fine carborundum powder or with a fine nylon brush.
- (2)** Set up 50 ml of digestion medium (for 10 - 15 g of plant tissue) according to the recipe listed below (Solution A).
- (3)** Incubate the leaf slices or pieces in a 19 cm-diameter dish containing the digestion medium for 3 hours at 25 °C, covered with a plastic film. It may be advantageous to replace the digestion medium at intervals of 1 hour, as the enzymes might become inactivated by substances released from broken cells.
- (4)** After completion of the incubation the digestion medium is carefully removed and discarded. It normally contains very few protoplasts. The plant tissue is then washed 3 times by shaking gently with 20 ml wash medium (Solution B).
- (5)** After each wash the tissue is collected by pouring through a tea strainer (0.5 to 1 mm pore size) and the combined washes are then filtered through nylon mesh (100 -200 µm pore size) to remove vascular tissue and undigested material.
- (6)** The protoplasts are collected by centrifugation of the combined filtered washes for 3 minutes at 50 - 100 x g, aspirate and discard the supernatant.
- (7)** This crude protoplast preparation also contains some cells and chloroplasts and it is important to purify the protoplasts to remove these contaminants. This can be done with solutions of sucrose and sorbitol of different densities.
- (8)** The protoplast pellet is gently resuspended in 40 ml of Solution C and this suspension is divided among two 100 ml centrifuge tubes.
- (9)** To each tube add slowly 5 ml of Solution D and then overlay this with 5 ml of wash medium (Solution B) to make a three-step gradient.
- (10)** Centrifuge at 300 g for 5 minutes.
- (11)** The protoplasts now collect as a band at the interface between the 2 top layers. Carefully remove them with a Pasteur pipet.
- (12)** The protoplasts should be examined with a light microscope to ensure that the preparation is free of cells and chloroplasts.
- (13)** When a large portion of the protoplasts is pelleted in this sucrose/sorbitol gradient the density of the 2 layers can be increased by adding 5 - 10 % Dextran (15,000 -20,000 Mr) or 10 - 20 % Ficoll to increase the percentage of the floating protoplasts.

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- (14) The purified protoplasts can be concentrated by diluting with 10 ml of Solution B, centrifuging at 100 g for 3 minutes and then resuspending the pellet in a small amount of medium by gently shaking the tubes.

The protoplasts are stable for up to 24 h when stored on ice.

- (15) Photosynthetic activity of the protoplasts can be determined by measuring co-dependent O<sub>2</sub> evolution with an oxygen electrode, provided rapid stirring is avoided as this will break some of the protoplasts. A suitable medium is listed as Solution E.
- (16) Protoplasts exhibit a relatively broad pH optimum but at more acidic pH values the bicarbonate concentration should be lowered.

#### Solution A (Digestion medium)

Composition		For 50 ml use:
500 mM	D-Sorbitol	4.56 g
1 mM	CaCl <sub>2</sub>	7.35 mg
5 mM	MES-KOH, pH 5.5	49.00 mg
2 %	Cellulase «Onozuka» R-10	1.00 g
0.3 %	Macerozyme R10	0.15 g

*The pH must be adjusted to 5.5 with KOH before adding the enzymes.*

#### Solution B (Wash medium)

Composition		For 100 ml use:
500 mM	D-Sorbitol	9.11 g
1 mM	CaCl <sub>2</sub>	14.70 mg
5 mM	MES-KOH, pH 6.0	98.00 mg

*The pH of the solution must be adjusted to 6.0 with KOH.*

#### Solution C

Composition		For 100 ml use:
500 mM	Sucrose	8.56 g
1 mM	CaCl <sub>2</sub>	7.40 mg
5 mM	MES-KOH, pH 6.0	49.00 mg

*The pH of the solution must be adjusted to 6.0 by adding KOH.*

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#### Solution D

Composition		For 100 ml use:
400 mM	Sucrose	6.80 g
100 mM	D-Sorbitol	0.90 g
1 mM	CaCl <sub>2</sub>	7.40 mg
5 mM	MES-KOH, pH 6.0	49.00 mg

*The pH of the solution must be adjusted to 6.0 by adding KOH.*

#### Solution E

Composition		For 100 ml use:
500 mM	D-Sorbitol	9.10 g
1 MM	CaCl <sub>2</sub>	15.00 mg
30 mM	Tricine-KOH	538.00 mg
5 mM	NaHCO <sub>3</sub>	42.0 mg

#### Reagents

Cat. no.	Product
16419	Cellulase «Onozuka» R-10
28302	Macerozyme R10
29834	MES (Morpholinoethane sulfonic acid)
30180	Sodium bicarbonate (NaHCO <sub>3</sub> )
35579	Sucrose
37195	Tricine (N-Tris(hydroxymethyl)-methylglycine)

#### Related Products

Cat. no.	Product
21373	Ficoll® 400
22168	Gelrite®

#### References:

- 1.) S.C. Huber and G.E. Edwards, *Physiol. Plant.* **35**, 203 (1975)
- 2.) S.P. Robinson et al., *Methodol. Surv. Biochem.* **9**, 13 (1979)
- 3.) G.E. Edwards et al., *Plant Physiol.* **62**, 313 (1978)
- 4.) T.L. Barsby et al., *Plant Cell Reports* **5**, 101 (1986)
- 5.) S.P. Robinson, *Methods Enzym.* **148**, 188 (1987)