VACUOLE ISOLATION
(This protocol was placed on the electronic Arabidopsis bulletin board; March 1992.)
From Marie-Theres Hauser, as used by Herman Hofte

* This is a vacuole prep protocol that worked very well in our hands both for Arabidopsis and tobbaco protoplasts (see note below).

1. SOLUTIONS
   (i) Lysis buffer
       0.2 M Mannitol
       10% Ficoll (400)
       20 mM EDTA
       2 mM DTT
       10 mM HEPES
       pH 8.0, adjust using NaOH
       Add 0.5 % BSA before use, prewarm to 42°C.
   (ii) Vacuole buffer
       0.5 M mannitol
       10 mM HEPES
       pH 7.5, adjust using NaOH
       protease inhibitor cocktail (0.5mM PMSF, 0.1 ug/ml leupeptin, 10-7M pepstatin)
       1mM EDTA
       150 ug/ml BSA
       * For tobacco, use 0.6M betain in place of 0.5M mannitol.

2. PROCEDURE
   (i) Prepare protoplasts. Make sure your protoplasts are completely digested (i.e., round).
   (ii) Make up 4% Ficoll solution. Mix 1 vol lysis medium with 1.5 vol vacuole buffer (3 ml/gradient). Add BSA (0.5% final) to lysis medium, prewarm to 42°C.
   (iii) Place 2-5x10^6 protoplasts in a glass tube, spin 5 min@500rpm.
   (iv) Remove most of the supernatant, resuspend protoplasts in remaining liquid.
   (v) Swiftly add swiftly 5 ml prewarmed (42°C) lysis buffer, pipette the solution 2-3 x up and down (wide bore pipette), transfer to small glass tube.
   (vi) Check under microscope, vacuoles should have come out, chloroplasts should be spread all over the place. If lysis is not complete, try repeating the agitation using the pipette.
   (vii) Layer 3 ml 4% Ficoll solution on lysis buffer and 1 ml of vacuole buffer. (4% Ficoll solution made with 1 vol lysis buffer (no BSA) and 1.5 vol vacuole buffer). Spin for 20 min@3,000 rpm in a swing out (RC-3 or HB4) rotor.
   * Vacuoles should concentrate at the interphase between the 4 %
Ficoll and the vacuole buffer. Collect vacuoles with a Pasteur pipette.

* Depending on the physiology of the plant the vacuoles can be more or less dense. You will, therefore, often find vacuoles also at the interphase between the 10% and the 4% layer.