Isolation of chloroplast-nuclei (nucleoids) from tobacco leaves

1. Collect 10 mature leaves of tobacco (20 – 30 cm in length, total 40 g fw).
2. Remove major veins and cut the leaf blade into 1 – 1.5 cm squares.
3. Homogenize the cut leaf blade in the isolation buffer (TAN buffer: 0.5 M sucrose, 20 mM Tris-HCl pH 7.6, 0.5 mM EDTA, 7 mM 2-mercaptoethanol, 1.2 mM spermidine, 0.4 mM PMSF) containing 0.1% (w/v) BSA with a Warling blender.
4. Filtrate the homogenate through Miracloth and nylon mesh with 50-μm pores.
5. Centrifuge the filtrate for 5 min. at 1400 g and 4°C. Discard the supernatant.
6. Gently resuspend the pellet in 40 ml of isolation buffer containing 40% (v/v) Percoll. Filter the suspension through a sheet of nylon mesh with 20-μm pores.
7. Overlay the filtrate (7 ml each) onto a cushion of isolation buffer containing 80% (v/v) Percoll (4 ml each) prepared in six 13-ml tubes (13-PA, Hitachi), and centrifuge the gradient for 30 min. at 110000 *g* and 4°C with a swinging-bucket rotor (RPS 40T, Hitachi).
8. Collect the green bands of intact chloroplasts. Dilute the chloroplast suspension to 150 ml with the isolation buffer and centrifuge it for 10 min. at 1000 *g* and 4°C.
9. Resuspend the chloroplast pellets in 12 ml of isolation buffer. Filter the suspension through a sheet of nylon mesh with 20-μm pores.
10. Overlay the filtrate onto a discontinuous sucrose density gradient (2.5 ml each of 80%, 40%, and 20% sucrose in isolation buffer) prepared in four 13 ml tubes (13-PA, Hitachi) and centrifuge the gradient for 30 min. at 6500 *g* and 4°C with a swinging-bucket rotor (RPS 40T, Hitachi).
11. Recover the green bands of purified chloroplasts at the 80%-40% sucrose interface. Dilute the suspension to 60 ml with isolation buffer and filter the suspension through a layer of nylon mesh with 20-μm pores. To the filtrate, add 60 ml of sucrose-free isolation buffer.
6. Incubate the diluted filtrate for 2 min. at 26°C, and add 6 ml of 20% Nonidet P-40 to the filtrate with continuous stirring for solubilization of the membrane system of the chloroplasts. Stir the mixture for 15 min. at room temperature.
7. Chill the mixture on ice. Centrifuge the mixture for 15 min. at 4400 *g* and 4°C to sediment debris. Recover the supernatant and filter it through a layer of nylon mesh with 5-μm pores.
8. Centrifuge the filtrate for 60 min. at 38000 *g* and 4°C to sediment chloroplast-nuclei. Suspend the pellet (chloroplast-nuclei) in 400 μl of isolation buffer with the aid of a 1-ml syringe equipped with a 27-G needle.
9. Determine the DNA content fluorimetrically with a DNA fluorometer (DyNa Quant, GE healthcare), and adjust the DNA concentration to the desired value. Dispense the sample into aliquots, freeze them quickly with liquid nitrogen, and store them at -80°C until required for further use.