Protocol of mtDNA isolation from cucumber

1. Ahead of time, place plants in dark for 3 days prior to isolation. The day before isolation, prepare the 2xwash solution and the homogenization solution without cysteine in advance and keep them at 4°C. Place 12 250-ml GSA tubes (large), 4 30-ml Corex glass tubes, and the blender container in the refrigerator to cool.

Homogenization buffer (modified from Holford et al. 1991)

TES	10 mM	229g/Mol	2.29 g/l
Sucrose	500 mM	342g/Mol	171.1 g/l
EGTA	10 mM	380 g/Mol	3.8 g/l
BSA	0.2%		2 g/l
Cysteine	0.05%		0.5 g/l – add later

Add 1 g KOH and then adjust pH with the 1M KOH solution. It takes a long time for the pH to equilibrate to 7.2

2X wash buffer (modified from Eubel et al. 2007)

Sucrose	500mM	342 g/Mol	171.1 g/0.5l
TES	10mM	229 g/Mol	2.29 g/0.5 l
BSA	0.1%		1 g/0.5l

Add 1 N KOH to adjust pH to 7.2

Buffer D (Lysis solution)

For 1L final volume:

- 25 g SDS
- 0.5 g Proteinase K
- 50 ml 1.0 M TRIS pH 8.0 (50 mM/L)
- 20 ml of 0.5 M EDTA pH 8.0 (10 mM/L)

2. Turn on the centrifuges (Sorvall RC-5B, Du Pont Instruments, and Beckman JA-13) at least one hour before using it. Put the GSA rotor in the Sorvall and the JS 13.1 swinging-bucket rotor into the Beckman.
3. Weigh 125 g of dark-treated tissue and 0.25 g cysteine
4. Add the tissue to 0.5% bleach solution with ice and set aside.
5. Prepare the Percoll step gradients. For this purpose, prepare 2 tubes of step gradient with 14%, 28% and 40% Percoll, using approximately 8-10 ml of 14% Percoll, 8-10 ml of 28% Percoll and 5 ml of 40% Percoll. Prepare the 1X wash buffer diluting a volume of 2X wash buffer with the same amount of water.

50% Percoll (26 ml)	100% Percoll 13 ml	+	2X wash buffer 13 ml
40% Percoll (10 ml)	50% Percoll 8 ml	+	1X wash buffer 2ml
28% Percoll (30 ml)	50% Percoll 16.8 ml	+	1X wash buffer 13.2 ml
14% Percoll (20 ml)	28% Percoll 10 ml	+	1X wash buffer 10 ml

6. Using 30-ml Corex glass tubes with orange rubber liners, add the 14% Percoll first, then 28% and 40% successively (use long glass Pasteur pipet and expel the liquid slowly at the bottom of the tube). Save the gradients at 4°C until needed.
7. Wash the plant tissue with tap water several times to eliminate the bleach and then at least 3 times with distilled water.
8. Add the 0.25 g cysteine to 500 ml of homogenization buffer. Then put the solution in the blender container with the tissue. Blend 5 times in the low setting for about one second.
9. The homogenized tissue is filtered through 8 sheets of cheesecloth and 2 of Miracloth. Put 4 single sheets of extra cheesecloth lining the inner part (to squeeze the sheets at the end of filtering to get the liquid get through easily). Filtering was done in a Büchner funnel and a side arm filtering flask under vacuum. Flask is kept on ice all the time.
10. The filtrate was put in 4 250-ml bottles and centrifuged a GSA rotor (Sorvall) twice at 3,500 rpm (2,000 xg) for 10 min. Each time bottles were changed and supernatant with mitochondria saved.
11. Centrifuge the bottles at high speed (9,500 rpm = 15,000 xg) for 15 min for precipitating the mitochondria.
12. Discard the supernatant, gently resuspend the green precipitate with a soft paint brush, add 10 ml per tube (total 40 ml) of 1X wash buffer and put into 2 40-ml Oakridge plastic tubes for the SS34 rotor.
13. Centrifuge once at 3,500 rpm (1,500 xg) for 5 min
14. Transfer the supernatant to 2 clean Oakridge tubes and centrifuge 15 min at 14,000 rpm (23,000 xg)
15. Discard the supernatant and gently resuspend the pellet with a soft paint brush. Transfer the suspension with a polyethylene transfer pipette. Add about 0.5 ml 1X wash buffer to help with the transfer. The transferred supernatant was put slowly on top of the percoll gradient in each of the 2 glass tubes.
16. Centrifuge at 13,000 rpm (26,000 xg) in JS13.1 swinging-bucket rotor in the Beckman 2-21M centrifuge for 1 hour at 4 C.
17. Check for the presence of whitish band in the 28% Percoll step. Take it out using a plastic transfer pipette. Add 1X wash buffer in a volume 4X the amount of the recovered liquid in an Oakridge (40 ml) tube and centrifuge it at 14,000 rpm (23,000 xg) in the SS34 rotor during 15 min.
18. Discard the supernatant and retain the loose pellet and add again about 10 ml of 1X wash buffer and repeat the centrifugation at 14,000 for 15 min
19. Discard the supernatant with a Pasteur pipette and take the loose pellet with micropipette adjusted up to 100 µl in order to measure the volume.
20. Add 2 µl of **DNase** solution (150 µl 2X Wash buffer no BSA and 50 mg DNase I) and 100 µl 1M $MgCl_2$ on each ml of mitochondria suspension. Incubate on ice for 1 h.
21. The supernatant is discarded and the loose pellet is resuspended in 10 mls of Buffer D (**lysis buffer**) preheated to 37C; carefully resuspend mitochondrial pellet. Try to place all **mitochondrial lysis** suspensions in a single Oakridge tube. Allow lysis to occur for 1 hour at 37 degrees with occasional gentle mixing (every 10-15 minutes).
22. Add 10 µl **RNase** (stock 10mg/ml) incubate in 37°C 1 h.
23. Add equal volume of phenol and invert gently 40 times, and spin in HS-4 swinging-bucket rotor for 20 minutes at 6,000 rpm (7,000 xg).

24. Add equal volume of chloroform:isoamyl alcohol (24:1) Invert gently 40 times, and spin again in HS-4 rotor for 20 minutes at 6,000 rpm.
25. Remove top (aqueous) phase, and place in clean Oakridge tube and repeat step 21 at least once more... if solution is still cloudy do again (Need to do at least 2 extractions with phenol and/or chloroform).
26. Add 0.1 volume 3.0 M NaOCl and gently mix. Then add twice the volume (DNA with NaOCl) of ice-cold 100% alcohol mix gently inverting 50 times, and place at -20 C overnight.
27. Collect precipitated DNA by centrifugation. Spin in HS-4 rotor for 30 minutes at 7,000 rpm (9,500 xg).
28. Decant EtOH, allow tube to dry. Add a small amount (10 ml) of 76% EtOH + 10mM NH_4OAc, allow to sit for 5 minutes, and decant (be careful NOT to lose pellet). Place tubes in front of Laminar flow hood for 20 minutes to dry.
29. Resuspend pellet in a small amount of TE 8.0. It helps to pre-heat TE 8.0 to 60 C and allow DNA to incubate in TE at 60 C for 1 hour, this should dissolve DNA. Transfer solution to microfuge tube and a quick spin in the microfuge will pellet debris.
30. Measure DNA concentration using spec.