Small-Scale Chloroplast Isolation Protocol

This protocol is based on the following two references:

- Kley et al (2010) Isolating intact chloroplasts from small Arabidopsis samples for proteomic studies. Analytical Biochemistry 398, p198. doi:10.1016/j.ab.2009.11.016
- Van Wijk et al. (2007) Isolation of Chloroplast Proteins from Arabidopsis thaliana for Proteome Analysis. Methods in Molecular Biology, vol. 335: Plant Proteomics: Methods and Protocols

Stocks

Stock	MW (g/mol)	Amount for 1L (g)	Amt to make	Grams per amt to make
1M HEPES, pH 7.5*	238.3	238.3 g	0.5 L	119.15
0.5M EDTA	292.2	146.1	0.1 L	14.6
5M DTT**	154.3		1 mL	0.772

*add ~100 KOH pellets per 0.5 L total to obtain correct pH

**fill 1.5mL tube nearly full with DTT, add ~0.5mL H2O & heat slightly to dissolve

1X chloroplast GW buffer (grinding and wash buffer)

Final concentration	MW	For 1 L	For 500 mL
50mM HEPES-KOH (pH 7.5)		50 mL of 1M stock	25 mL
5mM EDTA		10 mL of 0.5M stock	5 mL
0.3M sorbitol	187.2 g/mol	56.16 g	28.08 g
10mM NaHCO3	84.0 g/mol	0.84 g	0.42 g
0.5mM DTT*		100 uL of 5 M stock	50 uL

*add DTT just before use

Consider adding 5mM ascorbic acid or cysteine as additional reductants

Need around 75 mL per sample/prep (23 mL lysis + 3 washes of 10-15 mL each); ~750 for 10 preps

For 3 preps: make up 225 mL cpGW and add 22.5 uL of 5M DTT

-> Remove 4 mL and add 40uL HALT protease inhibitor (Thermo Fisher 78437) for final wash

5X chloroplast gradient buffer (for making Percoll gradients)

Final concentration	MW	For 1 L	For 30 mL
250mM HEPES-KOH (pH 7.5)		250 mL of 1M stock	7.5 mL
25mM EDTA		50 mL of 0.5M stock	1.5 mL
1.5M sorbitol	187.2 g/mol	280.8 g	8.4 g

Need 1.6 mL per sample/prep; ~ 16 mL for 10 preps

Lysis buffer (to lyse chloroplasts, from Klaas van Wijk)

Final concentration	For 1 L	For 10 mL
10mM HEPES-KOH (pH 7.5)	10 mL of 1M stock	100uL of 1M stock
5mM MgCl2	10 mL of 0.5M stock	100uL of 1M stock
Protease inhibitor 1X		

Not needed if only generating pelleted chloroplasts

Making Percoll gradients

Two solutions of Percoll (Sigma-Aldrich GE17089101) need to be made:
Percoll 40%
Percoll 80%

Make one gradient per plant sample (~2mL cp pellet).
Each gradient = 3 mL of 80% + 5 mL of 40% per gradient, 2 mL resuspended cp pellet
Total volume per gradient = 8 mL gradient + 2 mL cp = 10 mL so use culture tubes to prepare
Open Percoll in the laminar flow hood!

Solution	Vol for one gradient		**5x gradient buffer**	**Percoll**	**Water**
80% Percoll	3 mL		0.6	2.4	0
40% Percoll	5 mL		1	2	2

Gradient is most easily made by adding the 40% solution to the tube and underlaying 80% solution.

If running three samples, make up these solutions so there is plenty for 3 gradients

Solution	Vol for 4 gradients		**5x gradient buffer**	**Percoll**	**Water**
80% Percoll	3 mL		2.4	9.6	0
40% Percoll	5 mL		4	8	8

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Notes before starting

Grow plants under short day length for ~2 months
Cool all solutions to 4C before isolation
Put green lightbulbs into lamps in the cold room, green light helps reduce oxidative stress in cps
Put grinding equipment (mortar and pestle) in the cold room
Prepare funnels with 2 layers miracloth
Prechill rotors at 4C - swing bucket rotor and buckets for the ultracentrifuge
    Fixed angle large rotor and microfuge rotor for benchtop 4C centrifuge – Run this centrifuge for a few minutes to cool to 4C
Set up percoll gradients and keep in the cold room
Ideally perform protocol in the morning, a bit after the lights come on

Equipment

Green lightbulb and lamp
Transfer pipette for making gradients and pipetting cp band from gradient
Weigh boat
Large beaker to collect tissue into and rinse plant material
Salad spinner to remove extra water
Beaker covered in foil with foil top (to keep plant material in at 4C)
Small or medium size mortar and pestle
Miracloth (2 layers per prep; 6 inch squares works for small funnel; VWR EM475855-1R)
Small funnel
Spatula to squeeze out miracloth
Serological pipettes (10mL for loading gradient, 2 x 25 mL for cpWB and gradient waste)
Liquid waste container
Cut blue 1000uL tips for final wash
Cut green 200uL tips for taking aliquots
Paintbrush to resuspend pellet
Microcentrifuge tubes, pre-labled and stored at 4C (1 for total lysate aliquot; 1 for broken cp aliquot; 1 for final cp aliquot; 4 for final cps)
Container for holding liquid N and tongs for removing samples

Harvest and grind tissue

- Weigh out 2-3 g of leaf tissue and rinse in water to remove soil, insects, etc
- Spin out in salad spinner or shake to remove water
- Put leaves in plastic beaker on ice (cover beaker with foil or paper towel)

In the cold room

- Prewet miracloth (2 layers) in the funnel with a small amount of cpGW buffer (funnel sits on top of 50 mL falcon tube in an ice bucket)
- Add leaves to prechilled mortar (small or medium size) along with 23-25 mL of cpGW buffer
- Macerate tissue rapidly but thoroughly ~30-40 sec of grinding
- Pour ground leaf tissue and buffer through the miracloth and lightly squeeze with a spatula *(remove small aliquot ~60uL of total cellular lysate for future testing **this is total lysate sample sent for proteomics, with HALT protease inhibitor added before freezing)*
- Make sure the tubes contain roughly equal volumes
- Spin down the lysate at 1300 x g for 5 minutes in fixed angle rotor
- Decant supernatant, add ~2 mL of cpGW buffer and resuspend with paintbrush

Run gradient

- Load ~2mL of cp solution onto percoll gradient using a 10 mL serological pipette
- Place gradient into prechilled swing out rotor bucket (HS-4 with adapters)
- Centrifuge 10 min at 2500 x g in swing out rotor at 4C

Remove chloroplasts and wash

Take everything back to the cold room (green lights on).

- There should be a distinct band between the 40/80% percoll steps that contains intact chloroplasts
- Pipette off some of the top layer (containing broken cps with a 25 mL serological pipette) to reduce contamination *(remove small aliquot ~60uL of broken cps for future testing if desired)*
- Remove the chloroplast band at the 40/80 interface using a large volume transfer pipette and transfer to a cold ~15mL culture tube with lid
- Add 10 mL of cpGW buffer and mix by inversion
- Centrifuge 5 min at 1000 x g in a fixed angle rotor at 4C
- Decant supernatant and add ~1mL of cpGW to pellet
- Gently resuspend pellet with a NEW paintbrush (some typically pellets to wall of tube)
- Add ~13 mL of cpGW buffer and mix by inversion
- Centrifuge 5 min at 1000 x g in a fixed angle rotor at 4C

-Repeat the wash step one more time

- Resuspend chloroplasts in ~1mL of ***cpGW buffer WITH 1X protease inhibitor added*** (Halt Protease Inhibitor concentrate is 100X, so add 10 uL to 1mL solution)
- Transfer to a microcentrifuge tube using a cut blue tip – *(remove small aliquot ~60uL for future testing)*
- Check volume and separate into 4 equal amounts in microcentrifuge tubes (~250 uL each)
- Centrifuge 5 minutes at 1000 x g in 4C microfuge

(during this spin prepare liquid nitrogen container)

- Remove supernatant using a pipette and flash freeze cp pellet in liquid N_2; Store at -80C

Perform a protein Qubit on the aliquot of cp sample
Visualize fractions (lysate, broken cp, final cp) under phase contrast microscope at 20-40 X (cps should have a golden glow in the center, opacity suggests death)