# ISOLATION OF MAIZE CHLOROPLASTS FOR PROTEIN IMPORT STUDIES.

Mark Settles (modified from Ken Cline)

1. Bring trays of seedlings from growth chamber to bench. Let sit in ambient light for ~1 hour. This reduces starch accumulation in the chloroplasts. The seedlings should be grown 10-14 days in soil without fertilizer in standard maize growth conditions (78°F day, 65°F night, 18 hour days).

2. If using continuous gradients, make a Percoll gradient by mixing 14 ml **2 x GR** with 14 ml Percoll in a SW28 Ultraclear centrifuge tube. Establish gradient by spinning at 17,000 rpm in SW28 (52,000 x g) for 40'. Keep the gradient cold (4°C in centrifuge, ice on bench).

3. The seedlings should have about three leaves showing. Cut the seedlings to get as many of the younger leaves as possible. Chop 25 g of leaf tissue into 200-250 ml **GR** (ice cold). You need about 40 seedlings for 25 g of tissue.

4. Grind the tissue in a blender with 4 very short pulses on a low speed. Turn the blender on then off almost immediately.

5. Filter through 1 layer of pre-wet miracloth or cheesecloth. Squeeze gently to increase the yield.

6. Spin down chloroplasts at 3,000 g for 8'. A swing out rotor is probably best for this step.

7. Resuspend the pellet in 5 ml of **GR** (more than this will overload a 28 ml gradient) using a wide bore transfer pipette or a camel hair brush. Layer onto the Percoll gradient.

8. Spin at 6,000 rpm (6,500 x g) in the SW28 for 15'.

9. Remove the top band (broken plastids) and discard. Take the lower band and dilute three fold with **import buffer** (**IB**).

10. Spin down chloroplasts at 4,000 rpm (2,600 x g) for 8' in HB-4 rotor.
11. Resuspend the pellet in 15 ml **IB**.

12. Spin down the chloroplasts for 4' at the same speed as in 10.

13. Resuspend in an appropriate volume of **IB** (~1 ml of **IB** per 25 g tissue) to get ~1mg chlorophyll per ml.

14. Test chlorophyll concentration. Make a 1:10 dilution of the chloroplasts in **IB**. Mix the following in four tubes

| Water | Chloroplasts | Acetone |
|---|---|---|
| 200µl | 0µl | 800 µl |
| 160µl | 40µl | 800 µl |
| 140µl | 60µl | 800 µl |
| 120µl | 80µl | 800 µl |

Take the $OD_{652}$ of each tube. If the OD is above 1 dilute the chloroplasts more to get an accurate measure. Calculate chlorophyll concentration with the following formula:

mg/ml = $(OD_{652})(0.02899)(1000/\text{ml of chloroplasts})(\text{dilution factor})$

**GR Buffer (1L)**

| | |
|---|---|
| 50 mM Hepes-KOH pH7.5 | 50 ml 1 M Hepes |
| 0.33 M Sorbitol | 100 ml 3.3 M Sorbitol |
| 1 mM $MgCl_2$ | 1 ml 1 M $MgCl_2$ |
| 1 mM $MnCl_2$ | 1 ml 1 M $MnCl_2$ |
| 2 mM EDTA | 2 ml 0.5 M EDTA |
| 5 mM Na-ascorbate | 1 g Na-ascorbate |
| 1% BSA | 10 g BSA |

Add the Na-ascorbate and BSA fresh the day you use the GR

**Import Buffer (1L)**

| | |
|---|---|
| 50 mM Hepes pH 8.0 | 50 ml 1 M Hepes |
| 0.33 M Sorbitol | 100 ml 3.3 M Sorbitol |

If you are using step gradients, use an 85% step and a 35% step. For a 30 ml tube pour 6 ml 85% and 15 ml 35%. Increase these volumes if you are loading more than 3 ml on the gradient. The steps should be in GR.