BUNDLE SHEATH AND MESOPHYLL CELL PREP.

Mesophyll Cell Prep (Modified from Sheen) (Do this one first)

1. Cut up to 5 g of leaves perpendicular to the midrib in 0.5 to 1 mm strips.

2. Add to 80 ml **Enzyme Buffer** in a flask with a side arm.

3. Apply a vacuum until all sections are infiltrated.

4. Transfer to a large petri dish and digest for 3 hours at room temp.

5. Discard the broken cells by filtration through a 135 μM mesh (Millipore). Resuspend the residual partly digested segments in 50 ml **Wash Buffer** in the same petri dish.

6. Press the leaf strips very gently with a spoon, rocking it back and forth to release the protoplasts.

7. Filter through 60 μM nylon mesh. To make a filter, cut a 50 ml Falcon tube in half and cut out the centre of the cap. Place the mesh in the cap and screw onto the tube. Pour the leaf bits into the tube.

8. Pellet cells by centrifugation at 300 g for 5' in a benchtop centrifuge.

9. Resuspend cells in 25 ml **Wash Buffer** and then pellet again.

10. Resuspend in 500 μl of **Wash Buffer**. For RNA preps, pipette into a plastic peel away mold, drop into liquid nitrogen and store at –70°C.

ENZYME BUFFER (100ml)

20 mM Mes pH 5.5	2 ml 1 M
1 mM $MgCl_2$	100 μl 1 M
0.6 M sorbitol	30 ml 2 M
2% Cellulase Onozuka	2 g
0.1% macerase	0.1 g

WASH BUFFER (100ml)

50 mM Tris pH 7.5	2.5 ml 2M
1 mM $MgCl_2$	100 μl 1 M
0.6 M sorbitol	30 ml 2 M
100 mM β-mercaptoethanol	694 μl

(Calbiochem)

Bundle Sheath Prep (modified from Westhoff)

1. Cut four leaves (3rd leaf as 4th emerging) into 2 mm x 2 mm squares

2. Put into a chilled blender with 50 ml **Bundle Sheath Buffer 1**

3. Give 3 x 10 sec. pulses at low speed.

4. Filter through a 60 μM mesh. To make filter, cut a 50 ml Falcon tube in half and cut out the cap. Place the mesh in the cap and screw onto the tube. Pour the leaf bits into the tube.

5. Pour 35 ml **Bundle Sheath Buffer 2** back through net to return leaf debris into blender.

6. Blend at high speed 1 min.

7. Filter through 60 μM mesh as in 4.

8. Repeat steps 5 through 7 three times.

10. Freeze final filtrate in liquid nitrogen on net. Keep cells at –70°C.

Bundle Sheath Buffer 1 (100 ml)

0.33 M sorbitol	6 g
0.3 M NaCl	6 ml 5 M
0.01 M MgCl2	1 ml 1 M
0.01 M EGTA	2 ml 0.5 M
0.001 M aurintricarboxylic acid	42 mg
0.01 M DTT	154 mg
0.005 M DECA	500 μl 1 M
0.2 M Tris pH 9.0	10 ml 2 M

Bundle Sheath Buffer 2 (500 ml)

0.35 M sorbitol	31.8 g
0.005 M EDTA	5.0 ml 0.5 M

0.001 M aurintricarboxylic acid	210 mg
0.1% β-mercaptoethanol	500 µl
0.05 M Tris pH 8.0	25 ml 2 M