FLAGELLA PREP PROTOCOL
Witman, 1986.*

1. Begin by making all solutions fresh for each prep. KCl can be saved and does not need to be made fresh. Store HMDS, HMDS-EGTA, and HMDS-25% on ice. Store all other solutions at 4°C.

2. Be sure to wear gloves, and if necessary mask and lab coat during prep so as to avoid sample contamination with keratin and proteases.

3. Harvest cells in Sorvall RC-3B refrigerated centrifuge using (6) 250-mL bottles at 2000 RPM for 5 min at RT. Rotor for this experiment is H-6000A. Remove supernatant by aspiration and resuspend immediately in 10 mL 10mM HEPES pH 7.5 at RT. Transfer resuspended cells to 50-mL Nalgene conical tubes (#3105-0050). Combine up to 3 cell concentrates and bring total volume to 45 mL with 10 mM HEPES in each conical tube.

4. Centrifuge at 2000 RPM for 5 min at RT. Aspirate off supernatant and wash with 10 ml 10 mM HEPES at RT. Combine tubes to get a max of 4 mL packed cell volume per 50-mL Nalgene conical tube. Bring total volume to 45 mL with 10 mM HEPES at RT.

5. Centrifuge as in step 4. Resuspend cells in ice-cold HMDS and bring total volume HMDS+cells to 10 mL. Set RC-3B centrifuge to 4°C.

6. Add 2.0 mL Dibucaine (25 mM) to each of the resuspended tubes and pipette up and down with a 10 mL plastic pipette or transfer pipet. After 2 min of periodic pipetting, check under phase-contrast microscope to see if flagella have been removed.

7. If flagella are detached, quickly add 28 mL ice-cold HMDS-EGTA to conical with cells, one 28-ml volume to one volume of cells. Centrifuge at 2500 RPM for 5 min at 4°C with brake set to ZERO.

8. Collect ~35-mL supernatant, and distribute into clean 50-mL Nalgene conical tubes. Underlay each supernatant collection with 9 mL HMDS-25%. Centrifuge at 2800 RPM for 10 min at 4°C.

9. Collect supernatant and distribute into 50-mL Nalgene round bottom tubes (#3117-9500). Centrifuge at 16,000 RPM in SS-34 Sorval Rotor for 20 min at 4°C. Discard Supernatant.

10. Resuspend flagella in ~1.0 mL ice-cold HMDEK and save ~0.1 mL of whole flagella if continuing on to strip the membrane and matrix fraction. If flagellar pellet is large, increase HMDEK to 2.0 mL and remove 0.2 mL whole flagella. We have been adding a protease inhibitor cocktail to this final HMDEK buffer.

For production of Membrane + Matrix fraction:

12. Add 0.1 mL 10% NP-40 to 0.9 mL remaining resuspended flagella in HMDEK (0.2 mL 10% NP-40 to remaining 1.8 mL for large pellet). Pipette up and down gently and allow sample to sit on ice for ~15-30 min. Centrifuge samples at 16,000 RPM in SS-34 Sorval rotor at 4^{o}C for 20 min.

13. Collect and store supernatant containing 'Membrane + Matrix' fraction. Resuspend axonemes in 1 mL ice-cold HMDEK+ protease inhibitor cocktail. Store both fractions at 4^{o}C until needed.

For production of KCl Extract and KCl-extracted Axonemes:

14. After removal of M+M, and resuspension of axonemes in 1 mL of HMDEK, add 1 mL 1.2M KCl for a working concentration of 0.6 M KCl. Stir and pipette up and down gently. Allow to incubate on ice 15-30 min. Centrifuge samples at 16,000 RPM in SS-34 Sorvall rotor at 4^{o}C for 20 min.

15. Collect and store KCl wash fraction, and resuspend KCl extracted axonemes in 0.5 – 1 mL HMDEK+ protease inhibitor cocktail. Store at 4^{o}C.

* Witman, G. B. 1986. Isolation of *Chlamydomonas* flagella and flagellar axonemes. **Meth. Enzymol.** *134*: 280-290.

FLAGELLA PREP PROTOCOL
BUFFERS

Solutions and Buffers*:*

10mM HEPES ph 7.5	**make up 1 liter**	**must make FRESH**
1 M HEPES ph 7.5	10 ml	**from -20°C stocks**
H2O, nanopure water	990 ml	
DTT-1 M	**make up ahead**	**located in -20°C**
DTT (MW 154.2)	1.54g/10ml dH20	
HMDS		
10 mM HEPES, 5mM MgSO4, 4% Sucrose, 1mM DTT		**must make FRESH**
1M HEPES	10 ml	**ICE**
1M MgSO4	5 ml	**make up 1 liter**
Sucrose	40 g	
DTT	1 ml stock	
dH2O		
HMDS-EGTA	**make up 4 tubes for prep**	**must make FRESH**
HMDS	28 ml per tube from above	**ICE**
0.1 M EGTA	140 µl per tube	
HMDS-25% Sucrose	**make up 100 ml**	**must make FRESH**
HMDS	100 ml from above stocks	**ICE**
Sucrose	25 g	
25mM Dibucaine-HCL	**make up 15ml**	**must make FRESH**
Dibucaine (MW 379.9)	0.1425 g	
dH20	15 ml	
HMDEK		
30 mM HEPES, 5 mM MgSO4, 1 mM DTT, 0.5 mM EGTA, 25 mM KCL		
1 M HEPES	30 ml	**must make up FRESH**
1 M MgSO4	5 ml	**ICE**
DTT	1 ml stock	**make up 1 liter**
0.1 M EGTA	5 ml	
1 M KCL	25 ml	
dH2O		

10% Nonidet P-40

10% Solution NP-40 Alternative purchased from Calbiochem #492018 50 ml
Long-term storage 4ºC

1.2 M KCl		
KCL (MW 74.55)	8.95 g in 100 ml dH2O	**can make up ahead**