WORKSHEET
HMM preparation

Number:
Myosin preparation used:

Solutions, buffers:

Check:

1. BED — Volume: 100 ml _____

0.1 mM $NaHCO_3$	0.02 ml stock (0.5M)
0.1 mM EGTA	0.04 ml stock (0.25M)
1 mM DTT (add fresh from 1 M stock)	100 μl

2. 2xCHB — Volume 25 ml _____

20 mM imidazole-HCl (pH 7.0)	0.5 ml stock (1M)
1 M KCl	10 ml stock (2.5 M)
4 mM $MgCl_2$	0.05 ml stock (2 M)
10 mM DTT (add fresh from 1 M stock)	0.25 ml

3. PMSF stock — Volume: 10 ml _____

0.2 M PMSF	0.348 g
Ethanol	to 10 ml

4. Chymotrypsin — Volume: 2 ml _____

5 mg/ml TLCK-chymotrypsin	0.01 g
2xCHB	to 2 ml
OR:	Volume: 1 ml
0.5 mg/ml TLCK-chymotrypsin	0.5 mg
2xCHB	to 1 ml

5. Inhibitor buffer _____

3 mM $MgCl_2$	Volume: 25 ml 0.075 ml stock (1 M)	10 ml 30 μl (1 M)
0.1 mM PMSF	0.0125 ml stock (0.2 M)	5 μl (0.2 M)
BED	to 25 ml	to 10 ml

Steps:

1. Add 9 volumes of BED to stock myosin in a centrifuge tube, and mix to precipitate the filaments. (Slowly pipet buffer on myosin while vortexing.) Incubate on ice for >10 min. _____
Volume of myosin:
Total volume:

2. Centrifuge at low speed in a swinging bucket rotor. _____
(2-4000 rpm, 30 min, 0 ˚C)

3. Dissolve the pellet in 2xCHB, and add BED as needed to achieve a final concentration of ~15 mg/ml myosin. (Typically, one-to-one volume ratio) _____
Volume of pellet (est.):
Volume of added 2xCHB

4. Incubate myosin solution at 25ºC for 10 min. _____
(On water bath. Monitor temperature.)

5. Add TLCK-treated chymotrypsin to a final concentration of 12.5 μg/ml, gently mix the reaction and incubate _____
7.5 to 10 min at 25ºC.
Volume of added chymotrypsin stock:
Digestion time:

6. Add 9 volumes of ice cold inhibitor buffer to the reaction and mix. _____

(Slowly pipet buffer on protein while vortexing.)
Incubate on ice for 1 hour.

7. Centrifuge the suspension at high speed. _____
[Beckman 50Ti rotor, 40,000 rpm (~100,000 *g*), 30 min, 0 °C]

8. Store supernatant (typically 0.4-0.7 mg/ml, and >90% pure) on ice. _____
Use in the motility assay for 3-5 days. A_{280}=0.60 cm^2/mg.

SDS-PAGE:
Type of gel: Percentage: Sample preparation: Standard:

Loading: 1. 2. 3. 4. 5. 6. 7. 8. 9. 10.

Actin-affinity purification of HMM:
Solutions, buffers:
1. HMM (see above), concentration determined _____
2. F-actin (concentration determined). **Number** of Actin prep: _____
3. ATP stock (300 mM) _____
4. AB buffer (see *In vitro* assay worksheet) _____

Steps:
1. Dilute HMM in AB buffer to a final concentration of about 0.4 mg/ml _____
Volume of HMM:
Volume of added AB:

2. Add F-actin to a final concentration of 1.6 mg/ml. _____
Mix gently but thorougly.
Volume of F-actin solution:

3. Add ATP stock to a final concentration of 1 mM _____
(or to a final concentration not more than that used in the *in vitro* assay)
Mix genty but thoroughly.
Volume of ATP stock:

4. Incubate mixture on ice for 10 minutes _____

5. Centrifuge _____
[Beckman 50Ti rotor, 45,000 rpm (150,000 g), 30 min, 0 °C]

6. Use supernatant in the *in vitro* assay, diluted typically to 40-60 μg/ml.

Example:

Component	**Volume**	**Final concentration**
HMM (stock cc.: 0.67 mg/ml)	1.2 ml	0.4 mg/ml
F-actin (stock cc.: 5 mg/ml)	0.64 ml	1.6 mg/ml
ATP (stock cc.: 300 mM)	6 μl	1 mM
AB buffer	0.154 ml	
Total volume:	**2 ml**	