Preparation of and *in vitro* motility assay on human cardiac myosin

Specimen number:

Experiment number:

Buffers, solutions:

1. Myosin extraction buffer		**volume: 500 ml**
	0.3 M KCl	11.184 g
	0.09 M KH_2PO_4	6.124 g
	0.06 M K_2HPO_4	5.225 g (pH 6.8)
	1 mM $MgCl_2$	500 μl stock (1 M)
Fresh for 5 ml:	0.2 mM ATP	3.3 μl (300 mM stock)
	1 mM DTT	5 μl (1 M stock)
	0.23 mM PMSF	5.75 μl (0.2 M stock in EtOH)

2. AB buffer	**Volumes:**	**100 ml**	**250 ml**	**1000 ml**
25 mM imidazole-HCl (pH 7.4)		0.17 g	0.425 g	1.7 g
25 mM KCl		0.186 g	0.466 g	1.864 g
4 mM $MgCl_2$ (1 M stock)		0.4 ml	1 ml	4 ml
1 mM EGTA (0.5 M stock)		0.2 ml	0.5 ml	2 ml
1 mM DTT (fresh 1 M stock)		100 μl	250 μl	0.154 g

3. 0.6 M KCl/AB	**Volumes:**	**50 ml**	**100 ml**
0.6 M KCl		2.144g	4.287g
—add AB buffer—			

4. AB/BSA	**Volumes:**	**50 ml**	**100 ml**
0.5 mg/ml BSA		0.025 g or 125 μl stock (200 mg/ml)	0.05 g or 250 μl stock
—add AB buffer—			

5. GOC	**Volumes:**	**2 ml**	**5 ml**	**10 ml**
glucose stock*		40 μl	100 μl	200 μl
catalase**		4 μl	10 μl	20 μl
glucose oxidase***		16 μl	40 μl	80 μl
AB/BSA		1.925 ml	4.815 ml	9.63 ml
β-mercaptoethanol		15 μl	37.5 μl	75 μl

*glucose stock is 30% (1.665M) in water + NaN_3 stored at 4 ˚C. Final concentration of glucose is 3 mg/ml.
**catalase is prepared fresh, stock concentration 20 mg/ml in AB buffer.
***glucose oxidase is prepared fresh, stock concentration 25 mg/ml in AB buffer.

6. ATP	**Volumes:**	**1 ml**	**3 ml**	**Actual prep:**
1 mM ATP (from 300 mM stock)		3.3 μl	10 μl	
GOC		0.9967 ml	2.99 ml	

*ATP stock is 300 mM aqueous solution of ATP, pH 6.8, in 50 μl aliquots, quick-frozen in liquid nitrogen, and stored at -20˚C or, preferably, at -80 ˚C. Final concentration is adjusted as desired.

7. Actin	**Volume:**	**1 ml**	**5 ml**	**Actual prep:**
TRITC-Ph-F-actin stock		2 μl	10 μl ml	Prep #:
ATP		0.998 ml	4.99 ml	

*Note: dilute actin so that a approximate final dilution of 50,000 of the stored F-actin is obtained. Adjust to a concentration that produces an optimal distribution under the fluorescence microscope.

8. Myosin. Preparation depends on final myosin concentration. For the *in vitro* motility assay, 0.1-0.2 mg/ml myosin concentration should be used.

Experiment Steps: **Check:**

A. Preparation and storage of muscle sample:

1. Weigh human cardiac muscle sample. Weight =..... mg. _____
2. Take ~50 mg muscle and place in a 1.6-ml Eppendorf centifuge tube. Pierce the tube cap to prevent explosion during and after quick freezing. _____
3. In the Eppendorf tube, mince muscle into fine pieces with the tips of a pair of fine scissors. _____
4. Label the tube properly, then quick-freeze the tube in liquid nitrogen. _____
5. Store in -80 ˚C freezer until preparation of myosin. _____

B. Preparation of myosin:

1. Add 10 ml/g-muscle myosin extraction solution to the muscle (~0.5 ml).Buffer volume:.....ml. _____
2. Place muscle suspension into glass homogenizer, and homogenize for 30 min. on ice. _____
3. Incubate on Rotorack for 30 min. in cold room. _____
4. Centrifuge at 140.000g, 30 min, 4 ˚C. _____
5. Dilute supernatant 10-fold with 1 mM DTT in ddH_2O.
   Preparation of 1 mM DTT: add 20 μl DTT stock (1 M) to 20 ml water)
   Volume of supernatant: .....ml.
   Volume of 1 mM DTT added: .....ml. _____
6. Incubate on ice for 60 minutes (to precipitate myosin). _____
7. Centrifuge at 20.000g, 20 min, 4 ˚C. _____
8. Dissolve pellet in ~equal volume of 0.6 M KCl/AB solution.
   Volume of 0.6 M KCl/AB added (usually 100 μl): .....μl. _____
9. Measure concentration of myosin against 0.6 M KCl/AB as background.

$$[myosin](mg/ml) = \frac{OD_{280}}{0.53} \times (dilution)$$

   Myosin concentration = ...mg/ml. _____

10. Dissolve an aliquot of myosin in Fairbanks sample buffer _____
    Fresh Fairbanks sample buffer: 20 mg DTT in 1 ml 3x Fairbanks buffer
    2 volumes myosin + 1 volume Faibanks buffer, 1 min. at 90 ˚C. Quickfreeze 20 μl aliquots, store in -80 ˚C freezer.
11. Immediately carry out the *in vitro* motility assay. _____

C. Advance preparations for the *in vitro* motility assay:

1. Prepare flow cells (dip-coat 18 mm^2 coverslips with nitrocellulose (1% in amyl-acetate), and allow to dry overnight. _____
2. Place two Parafilm spacers (4x25 mm) on a precleaned microscope slide, position the 18 mm^2 coverslip on the top, and melt the Parafilm on a hotplate. _____
3. Store flow cells in a dust-free area. Use within 2-3 days. _____

D. The *in vitro* motility assay:

1. Turn on arc lamp (HBO200) ~10 min prior to experiments. _____
2. Turn on thermostat adjusted to an effective temperature of 37 ˚C (46 ˚C on thermostat) _____
3. Place the flow cell obliquely in a 50-ml beaker, and start adding the solutions: _____
   a. 10 μl myosin, wait 1 min.
   b. 100 μl AB/BSA.
   c. 100 μl actin in ATP/GOC solution
4. Oil the objective, and place the flow cell on the microscope stage.
   Wait for ~30 s for temperature to equilibrate. _____
5. Turn on the camera and bring actin filaments in focus. Adjust contrast and frame averaging with Argus-20 (usually 4 frames). Start video recording. _____

Temperature: **Tape Number:**

Telescope lens setting (default 2x): 1.25x 1.6x 2.0x

Video Recording:

Experiment	Step	Tape Time
1.	START	**h m s**
		h m s
	STOP	**h m s**
2.	START	**h m s**
		h m s
	STOP	**h m s**
3.	START	**h m s**
		h m s
	STOP	**h m s**
4.	START	**h m s**
		h m s
	STOP	**h m s**
5.	START	**h m s**
		h m s
	STOP	**h m s**

Measurement of filament velocity

1. Using NIH Image 1.61, grab a sequence of images (10-20 frames). _____
2. Carry our frame averaging if necessary. _____
3. Import the "In Vitro Motility Assay 2.3*" macro. _____
4. Execute the "Preprocess stack" command. _____
5. Make a rectangular ROI for a selected filament and execute the "AutoMeanFast" command. _____
6. Repeat step 5 for preferably each filament in the field of view. _____
7. Export the Results. _____
8. Import the Results text file into KaleidaGraph, and construct velocity histogram. _____

Spatial calibration for the 2x telescope lens setting (as of 2000/06/07): 6.374 pixels/μm

[For individual-frame measurements:
horizontal scale: 6.591 pixels/micron; vertical scale: 6.157 pixels/micron, pixel aspect ratio: 1.07]

Notes/Remarks/Modifications:

References:

1. Nguyen, T.-T.T., *et al*. *Circ*. *Res*. **79**, 222-226, 1996. (*in vitro* motility using human cardiac biopsy samples)
2. Kron, S.J., et al. *Methods Enzymol*. **196**, 399-416, 1991. (in vitro motility assay)
3. Margossian, S.S,. & Lowey, S. *Methods Enzymol*. **85**, 55, 1982. (skeletal myosin preparation)