

## Plasma Membrane ATPase Assay

1. Preincubate 200 $\mu$ l of ATPase mix at 37°C in a microfuge tube. ATPase mix made as described below.
2. Start the reaction by adding sample. I use 50 $\mu$ l of a gradient fraction. Do duplicate samples so you can average the result and do another tube that also contains 100 $\mu$ M vanadate. So three samples for each fraction.
3. Incubate for 10 min at 37°C.
4. Stop reaction by adding 40 $\mu$ l of 50% TCA (cold) and immediately place on ice.
5. Centrifuge for 3 min in microfuge in the coldroom.
6. Remove 200 $\mu$ l of supernatant and place in 13 x 100mm glass tubes. The remainder can be performed at room temp.
7. Add 1 ml of ammonium molybdate and vortex tubes. Let incubate for 10 min.
8. Add 1 ml of semidine and vortex. Let incubate 20-30 min.
9. Read A<sub>710nm</sub>. Blank the spec with sample buffer alone in a reaction tube.
10. Average the two values for the sample and subtract out the value of the (+) vanadate tube. This gives you the vanadate-sensitive ATPase value. This is compared to the standard to get the nmoles of phosphate released.

<u>ATPase Mix:</u>	<u>For 20 ml:</u>
5mM ATP	55.12 mg
5mM MgCl <sub>2</sub>	20.4 mg
10mM PIPES	69.26 mg
5mM NaN <sub>3</sub>	200 $\mu$ l of a 0.5M stock
5mM phosphoenolpyruvate, monopotassium salt	20.61 mg

Adjust to pH 6.7

Can be frozen in aliquots at this point. Before using in the reaction, add 5 $\mu$ l per ml of Sigma Type II pyruvate kinase to the mix.

### Ammonium molybdate:

Make a 10mM stock in water and store in the dark at room temp. For 200 mls use 2.472 g of ammonium molybdate.

### Semidine:

Semidine is really 4-Aminodiphenylamine. The stock is made by mixing 200 mg into 400 ml of 1% NaHSO<sub>3</sub> (4g sodium bisulfate in 400 ml water). The semidine does not go into solution well, so filter and store in the dark at room temp.

### Notes:

1. For a standard curve, add TCA to the same concentration as in the samples to allow formation of the end product. You can use a phosphate buffer to make the standard curve.
2. TCA causes slow hydrolysis of ATP which could increase the background. This is why once the reaction is stopped the tube is immediately placed on ice.
3. The vanadate stock solution is warmed in a boiling bath a few minutes prior to use to break up vanadate oligomers that form. The stock is stored in the dark and the solid is sodium orthovanadate, sold by Sigma.