

Nucleus Developer Notes

Published Jun 12, 2026

Correspondence to
Jillian Yujuico
jyujuico@calpoly.edu

Open Access

Copyright © 2026 Yujuico & Peterson. This is an open-access article distributed under the terms of the [Creative Commons Attribution 4.0 International license](#), which enables reusers to distribute, remix, adapt, and build upon the material in any medium or format, so long as attribution is given to the creator.

Open Source

The materials and processes described in this article are distributed under the terms of the [CERN Open Hardware License Permissive 2.0](#), which enables reusers to make and convey any component or device that apply the information made explicitly available under this license, so long as all notices are retained.

Combining Class I & Class II PPK2 in Synthetic Cells

Jillian Yujuico¹✉, and Zach Peterson¹✉

¹California Polytechnic State University, San Luis Obispo, CA, USA

Abstract

Characterize the enzyme kinetics of different enzyme variants involved within the PPK2 energy system family. The goal is to isolate and purify different polyphosphate-based enzymes then evaluate both their independent and encapsulated kinetic activity. Purification will be confirmed via SDS-PAGE. Enzyme activity will be measured via luminescence (ATP/ADP) assays, and protein expression in encapsulated systems via fluorescence and microscopy imaging.

Keywords PPK2, PURExpress, Liposome encapsulation, Polyphosphate, ATP regeneration

1. OVERVIEW

Characterize the enzyme kinetics of different enzyme variants involved within the PPK2 energy system family. The goal is to isolate and purify different polyphosphate-based enzymes then evaluate both their independent and encapsulated kinetic activity. Purification will be confirmed via SDS-PAGE. Enzyme activity will be measured via luminescence (ATP/ADP) assays, and protein expression in encapsulated systems via fluorescence and microscopy imaging.

2. BILL OF MATERIALS

Reagent	Product Name	Manufacturer	Catalog No.	Price	Storage Conditions	Link
POPC	16:0-18:1 PC 25 mg/mL	Avanti Lipids	850457C	\$435.00	-20C	16:0-18:1 PC (POPC) 26853-31-6 Avanti Research
Liss-Rhod-PE	16:0 Liss Rhod PE 1 mg/mL	Avanti Lipids	810158C-1mg	\$281.67	-20 C	16:0 Liss Rhod PE 384833-01-6 Avanti Research
Cholesterol	cholesterol (D7) 1EA	Avanti Lipids	A84100M	\$193.72	-20 C	cholesterol (D7) 83199-47-7 Avanti Research

Reagent	Product Name	Manufacturer	Catalog No.	Price	Storage Conditions	Link
Mineral Oil	Mineral oil, mixed weight	Thermo Scientific	AC415080010	\$53.40	RT	Mineral oil, pure 1 L Buy Online Thermo Scientific Chemicals thermofisher.com
Glucose	D-(+)-Glucose, 99%	Thermo Scientific	A16828.36	\$41.65	4C	D-(+)-Glucose, anhydrous, 99% 500 g Buy Online Thermo Scientific Chemicals thermofisher.com
PURE	PURExpress	NEB	E6800S	\$295.00	-80 C	PURExpress® In Vitro Protein Synthesis Kit NEB
RNase Inhibitor	RNase Inhibitor, Murine	NEB	M0314S	\$81.00	-20 C	RNase Inhibitor, Murine NEB
OptiPrep	OptiPrep™ Density Gradient Medium	Sigma Aldrich	D1556-250ML	\$373.00	4 C	OptiPrep Density Gradient Medium, 60 (w/v) iodixanol in water
DNA	pJL1-sfGFP				-20 C	
FTPPK2-I					-80 C	
AJPPK2-II					-80 C	

3. PROTOCOL

3.1. Part I – Protein Purification

Repeat for each enzyme class.

1. Add a 1g : 2ml ratio of cell pellet to S30 buffer
2. Supplement DTT to your buffer, 2 mM final concentration
3. Sonicate: 5x bursts of 10 sec on, 10 sec off @ 50% amplitude. Record total joules delivered *must keep tube ice cold during sonication
4. Centrifuge at 14,000rpm for 10 minutes @ 4C
5. Collect supernatant and transfer to a new tube, keep on ice
6. Perform spin-column as instructed via provided supplementary material
 - Use Buffer W for wash
 - Use Buffer BXT for elution

3.1.1. Spin Column Procedure – Adapted from iba by Cube Biotech Spin Column Kit Protocol – Version: PR137-0004. Revision date: 10.11.2025

- Strep-Tactin® XT 4Flow® high capacity Spin Column Kit
- 1. Resuspend Strep-Tactin® XT 4Flow® high capacity resin and pipet 100 µL of the 50% suspension into a spin column leading to a column bed volume of 50 µL
- 2. Centrifuge the sample (maximum speed, 5 min 4°C) to remove aggregates
- 3. Apply up to 500 µL sample to the spin column, close the column lid and incubate at room temperature with constant movement (rolling or shaking) for 5-30 min. For most purposes, 5 min are sufficient, but a longer incubation can increase the amount of captured protein, especially for large proteins (≥ 90 kDa)
- 4. After incubation, open the lid and break off the lower column seal. Place the spin column into a reaction tube and centrifuge for 30 sec at 500 x g.
- 5. Collect the flow-through for SDS-PAGE analysis and place the spin column into a new reaction tube. Apply 500 µL 1xBuffer W and centrifuge for 30 seconds at 700 x g (Optional: usually, one washing step is sufficient to obtain a highly pure protein, but this step can be repeated if higher purity is required)
- 6. Collect the washing fraction for SDS-PAGE analysis and place the spin column into a new reaction tube.
 - A. For fast processing, apply 150-200 µL 1x Buffer BXT, close the spin column lid and vortex briefly. After 5 minutes of incubation without movement, vortex again briefly, open the spin column lid and centrifuge for 30 seconds at 700 x g.
 - B. For maximum target protein concentration, apply 50 – 100 µL 1x Buffer BXT, close the spin column lid and vortex briefly. After 5 minutes of incubation without movement, vortex again briefly, open the spin column lid and centrifuge for 30 seconds at 700 x g. Repeat this step one to two times. At least 80% of the target protein will be in the first elution fraction.

3.2. Part II – PPK Screen and Standard Curve

1. Make a 1 mg/ml BSA solution from your 10 mg/mL BSA stock
 - 40 µL of 10 mg/mL BSA stock with 360 µL of DI water
2. Since stock concentration of PPKs is unknown, run a dilution screen for each protein class to identify which dilution falls within the standard curve. Incubate at room temperature for 10 minutes prior to running
 - See Table 1.
3. Make BSA standard curve solutions following Table 2. Run each standard in duplicate, incubating at room temperature for 10 minutes before running
 - See Table 2.
4. Compare A595 values to your standard curve. Identify the dilution factors whose A595 falls between S1 and S5, ideally near the midpoint.
5. Generate standard curve and calculate concentrations
 - Exclude blank from regression
 1. Plot A595 vs BSA concentration in µg/mL for S1-S5
 2. Fit linear trendline and calculate the concentration of each PPK2 using the trendline equation
 - Multiply by the dilution factor used in step 4 to get the original stock concentration

Sample	Dilution	Protein (μL)	DI Water (μL)	Bradford (μL)
PPK2 Class II (Elution 1)	1:2	6.25	6.25	37.5
PPK2 Class II (Elution 1)	1:5	2.5	10	37.5
PPK2 Class II (Elution 1)	1:10	1.25	11.25	37.5

Table 1. PPK2 dilutions.

Standard	1 mg/mL BSA (μL)	DI Water (μL)	Bradford (μL)	Final BSA concentration (μg/mL)
Blank	0	12.5	37.5	0
S1	1.56	10.94	37.5	125
S2	3.13	9.38	37.5	250
S3	6.25	6.25	37.5	500
S4	9.38	3.12	37.5	750
S5	12.5	0	37.5	1000

Table 2. Bradford dilutions.

3.3. Part III – SDS-PAGE

1. Add 6μL of your protein sample with 3μL of 4x Laemmli Dye and 4μL of water in a PCR tube
 - DI water is okay and in-fact preferred
2. Add 1μL of your 1M DTT into the PCR tube** (*DTT may have already been added*)
3. Incubate sample at 95 °C for 5 minutes to denature protein
4. Centrifuge your samples at max speed for 3 minutes.
5. Remove gel from packaging and take off bottom strip and comb on top
6. Place gel into the gel apparatus and ensure that it is sealed properly
7. Rinse wells with DI water first and then dab the wells with a Kim wipe and make sure to get all the water out of the well
8. Pour running buffer in the inner chamber until the buffer covers the wells
9. Fill the outer chamber until you reach the “two gel” mark with running buffer
10. Pre-run the gel at 150 V for about 10 minutes
11. Proceed to load 12μL of your protein sample into your gel including ladder
12. Run your gel at 200V for 40 minutes
13. Remove your gel cassette from the apparatus and image your results

3.4. Part IV – Activity Assays

3.4.1. ATP Assay

Materials:

- PPK2 Reaction Buffer: 50mM Tris-HCl, 10mM MgCl₂, 0.5mM DTT, and 0.1mg/mL BSA
- 2x Substrates Mix: 10mM AMP or ADP and 100mM PolyP
- CellTiter-Glo Reagent
- ATP Standard Solutions
- Opaque white 96-well plate
- 1.5mL tubes

Protocols:

Part A: Prepare reagents and reaction tubes

Before starting, thaw all reagents on ice and pre-equilibrate CellTiter-Glo 2.0 to room temperature (protect reagent from light).

1. Label two 1.5 mL microfuge tubes "A" and "B" for the duplicate reactions. Keep on ice.
2. Prepare the PPK2 Enzyme Mix:

20 μ L purified PPK2 enzyme [Enzyme Name: _____ Enzyme Class: _____] 180 μ L of PPK2 Reaction Buffer.

Pipet mix gently. Keep on ice.

1. Confirm the 2 $\times$ Substrate Mix is fully thawed, invert to mix.
2. *On ice:* prepare 6 microfuge tubes per replicate (A and B) for time-point aliquots by labeling:

Replicate A: A0, A1, A2, A4, A6, A10

Replicate B: B0, B1, B2, B4, B6, B10 (A/B = replicates; # = time point in minutes)

1. *On ice:* prepare 6 microfuge tubes per replicate for 1/10 $\times$ dilutions by labeling:

A0d, A1d, A2d, A4d, A6d, A10d

B0d, B1d, B2d, B4d, B6d, B10d

("d" denotes the diluted aliquot at that time point).

1. To each dilution ("d") tube, add 18 μ L of H₂O.

You will have a total of 24 tubes on ice.

Part B: Assemble the reaction master mix

Assemble the two replicate reactions in tubes A and B. Each reaction master mix is 150 μ L total. You will be removing the six time point aliquots from this tube. *Read steps 10–13 (Part C) before preparing the reaction master mix* You will be collecting time points so its essential that you are ready for next steps before you mix the enzyme with the substrate

Are you ready for steps 10–13?

7. To each of A and B (on ice):

add 75 μ L of PPK2 Enzyme Mix (from part A). Do NOT add the substrate mix yet.

7. Have the 2 $\times$ Substrate Mix, a timer, and a P200 set to 22 μ L ready.

Plan the order: you will add substrate to reaction A first, then reaction B, staggered ~15 seconds apart so each replicate runs its own timer.

7. Have all 12 time-point tubes (A0–A10, B0–B10) ready on ice

Reaction Master Mix Component	Volume per tube (μ L)
PPK2 Enzyme Mix	75
2 $\times$ Substrate Mix (ADP or AMP + PolyP)	75
For Class I arrow.r ADP	
For Combo Class I & II arrow.r AMP	
Total	150

Part C: Start the reaction and collect time points

10. Add 75 μ L of 2 $\times$ Substrate Mix to reaction A, pipet mix three times, start the timer, and IMMEDIATELY pull 22 μ L into tube A0 on ice. Repeat for reaction B (~15 s later), collecting into tube B0.

11. Incubate reaction tubes A and B in the 30 °C heat block. *Start your timers.*
12. At t (min) = 1, 2, 4, 6, and 10 minutes (per replicate clock), remove 22 μL aliquots from the reaction tubes A and B into the corresponding time-point tubes on ice (e.g., A1, A2, ... or B1, B2, ...).
13. After the $t = 10$ min sample is taken, place the reaction tube back on ice. The reaction is finished.
14. Prepare a 1/10 dilution:

For each time point aliquot (e.g., A0, A1, A2, ... or B0, B1, B2, ...), transfer 2 μL to the corresponding 1/10 dilution tubes (A0d, A1d, A2d, ... or B0d, B1d, B2d...) containing 18 μL H₂O.

10. Prepare ATP standard curve. ATP dilutions have been provided for you.
11. Transfer 20 μL of each ATP dilution to a new microfuge tube, label appropriately

Part D: Add CellTiter-Glo 2.0 and read

17. Using the 8-channel multichannel pipette and a reservoir (if available), dispense 20 μL of CellTiter-Glo 2.0 Reagent into each of the 24 tubes (A & B time points, 1x and 1/10 dilutions).

Goal: complete reagent addition for all 24 samples within 1–2 minutes.

17. Add 20 μL CellTiter-Glo 2.0 Reagent to each ATP dilution aliquot.
18. Briefly vortex each tube to mix. Cover with foil.
19. Incubate at room temperature in the dark for 10 minutes to equilibrate signal.

Part E: Load the plate

Use rows A through F of a 96-well white opaque plate per group (roughly one third of the plate). Example layout is:

Section	Wells used	Contents
1 $\times$ time course (Replicate A)	A1–A6	40 μL from A0–A10
1 $\times$ time course (Replicate B)	B1–B6	40 μL from B0–B10
1/10 $\times$ time course (Replicate A)	C1–C6	40 μL from A0d–A10d
1/10 $\times$ time course (Replicate B)	D1–D6	40 μL from B0d–B10d
ATP standard curve (8 points)	E1–E8	40 μL per standard (provided to you)
Buffer blank	F1	40 μL of 1 $\times$ PPK2 Reaction Buffer (no ATP, no enzyme)

21. Pipette 40 μL from each time-point tube into its assigned well
22. Pipette 40 μL of each ATP standard into the plate
23. Pipette 40 μL of 1 $\times$ Reaction Buffer into well F1 as a no-ATP blank.

Part F: Analysis

24. Read total luminescence on the GloMax Discover luminometer (*Protocol: CellTiter-Glo*). Read all wells in a single pass; do not pause between wells.
25. Record raw luminescence values for analysis.
26. Plot raw luminescence (y) vs [ATP] (x) for the standard curve on a log-log plot. Fit a linear region (typically spanning ~ 3 orders of magnitude).
27. For each time-point well, decide which dilution (1 $\times$ or 1/10 $\times$) falls within the linear range. Use that dilution to back-calculate [ATP] in the original reaction (multiply 1/10 $\times$ values by 10).
28. Plot [ATP] vs time for each replicate. Compute the mean and standard deviation across the two replicates at each time point.

29. Determine the initial rate (nM ATP / min) from the linear portion of the early time course (typically $t = 0$ to $t = 4$ min).
30. Using the protein concentration from Part II (Bradford assay), compute apparent specific activity in nmol ATP / min / mg protein.
31. Report whether the enzyme is active, with the supporting numbers and a qualitative comment on the consistency between the $1\times$ and $1/10\times$ readings.
32. Include all graphs in your notebook.

3.4.2. ADP Assay (Devised by Dr. Oza and Alyssa (Team 2))

Steps	Notes
1. Thaw all reagents on ice and pre-equilibrate CellTiter Glo 2.0 at room temperature	Wrap the Cell Titer Glo in foil to protect it from the light
2. Label the tubes as follows: PPK Enzyme Mix – A & B A replicates at time (minutes) – A0, A1, A2, A4, A6, A10 B replicates at time (minutes) – B0, B1, B2, B4, B6, B10 Dilutions of replicates A0d, A1d, A2d, A4d, A6d, A10d B0d, B1d, B2d, B4d, B6d, B10d (Total of 26 tubes, duplicate A&B)	Addition order Buffer ADP PPK No Dilutions were made
3. The addition of the 2X Substrate Mix is time sensitive, so only add them in the following order when ready PPK Master Mix: Purified PPK Enzymes – 20 uL Reaction Buffer – 180 uL PPK Master Mix: Add to both tubes (A&B) – 75 uL 2X Substrate Mix: Add to both tubes (A&B) – 75 uL	Stagger the addition of 2X Substrate Mix for tubes A & B by 30 – 60 secs
4. Prepare a timer and remove 22 uL at a time from the reaction tube during each time point: A0 = Reaction Tube A, Time: 0 minutes Record the actual time the sample was taken out and keep all samples on ice	Time Stamps: A0 A1 A2 A4 A6 A10 B0 B1 B2 B4 B6 B10
5. Make the dilutions for each aliquot by pipetting the following: All dilution tubes Aliquot - 2uL DI Water – 18 uL	No Dilutions were made

Steps	Notes
6. Add 50 uL of the Kinase Detection Reagent to each dilution aliquot	
7. Briefly vortex each tube to mix, cover with foil	
8. Incubate the tubes at room temperature in the dark for 10 minutes	
9. Into a 96 well plate, pipette the following: Aliquot tubes (6 in total) ATP Standards (4 wells) 1X Reaction Buffer (Blank) ADP-GLO Reagents Incubate for 40 mins	
10. Equilibrate the Kinase Detection Buffer + Kinase Detection Substrate at room temperature	Used premade Kinase Detection Reagent
Transfer the entire volume of the buffer into the substrate to make the Kinase Detection Reagent Mix by gently vortexing	
11. Add to the reaction wells in the plate Incubate for 40 minutes	
12. Record Luminescence	

3.5. Part V – Encapsulation

3.5.1. PURExpress Encapsulation

- Label three sets of 4 tubes, for each of the following:
 - E—oil emulsion (E1–E4)
 - T—transfer (T1–T4)
 - L—liposomes (L1–L4)

*1 refers to PURExpress only, 2 refers to Class I only, 3 refers to Class II only, and 4 refers to the Class mixture

- Create the PURExpress cytosol for encapsulation. Keep all reagents and reactions on ice throughout. Preparation on ice ensures that protein expression does not begin during assembly.
 - Pipette the following reagents in the tube labelled E in order from top to bottom using the appropriate pipettes.

Reagents (μL)	E1 (PURExpress)	E2 (Class I)	E3 (Class II)	E4 (Mix)
Nuclease-free H ₂ O	6.27	3.97	3.97	3.97
Solution A	26	26	26	26
Solution B	19.5	19.5	19.5	19.5
Enzyme	0	2.3	2.3	2.3
PolyP	4.2	4.2	4.2	4.2
Mg-Acetate	4.5	4.5	4.5	4.5

Reagents (μL)	E1 (PURExpress)	E2 (Class I)	E3 (Class II)	E4 (Mix)
RNAse Inhibitor	1.4	1.4	1.4	1.4
Optiprep	2.1	2.1	2.1	2.1
DNA (sf-GFP)	6.03	6.03	6.03	6.03
Total	70 μL	70 μL	70 μL	70 μL

- Pipette mix each reaction, then centrifuge the reaction at 5000g [6685 RPM for tabletop centrifuges (r=10 cm)] for 10-30 seconds to spin down bubbles in the reaction.
3. **Check the osmolarity** of your cytosol using a Vapor Pressure Osmometer before encapsulation.
 - Press “open” on the osmometer and use tweezers to place a circular sample disc on the reading area.
 - Pipette 10 μL of your cytosol onto the disc and press “close”.
 - Read and record the osmolarity.
 4. **Plate reader aliquots.** Pipette mix the cytosol solution before transferring 8 uL into two wells of a 384-well plate for kinetics quantification on the Synergy. Set the micropipette to 8 μL and **stop at the first stop** when dispensing to prevent the introduction of bubbles into the sample.

Encapsulate PURExpress into Liposomes

1. You will be given a range of glucose dilutions labeled with their concentration and osmolarity. Add 300 μL of the correct glucose outer solution, according to the osmolarity of your PURE cytosol, to the tube labeled T.
2. Add 150 μL of the lipid-oil mixture to the top of the PURE reaction in tube E.
3. Emulsify the lipid-oil and PURE mixture by running the tube along a row of empty slots on the 1.5 mL tube rack (watch instructional video for a visual aid on how to do this). Run it down 40–50 times until the solution forms a stable emulsion with an even milky color.
4. Slowly pipette the entire emulsion **down the side** of the corresponding T tube, so that the emulsion is layered on top of the glucose solution.
5. Centrifuge the T tube at 9000 g [8970 RPM for tabletop centrifuges (r=10 cm)] for 10 min at room temperature to pellet the liposomes.
6. Extract the liposomes from the T tube:
 - Remove the oil layer and lipid debris from the top (supernatant) of the T tube by gently pipetting with a 1000 μL pipette set to 200 μL. Begin at the top of the mixture and aspirate from the solution surface as the liquid level descends in the tube. Dispose of supernatant.
 - Once the majority of supernatant is removed, *extract liposomes by pipetting 50 μL of pellet and outer solution from tube T and transfer to the respective liposome tube L. Do not transfer the entire solution.* You now have synthetic cells!
7. Hold synthetic cells on ice in the L tube until you are prepared to begin measurement.
8. Pipet 30 μL of each sample into a 384-well glass bottom plate and transfer to microscope.
9. Once data is complete, perform analysis via ImageJ (Fiji) software.

3.5.2. Cytosol Encapsulation

Repeat the PURExpress Encapsulation steps above (Part V); however, do not follow the reagent table in step 2a; instead adhere to adjusted conditions below.

Reagents (μL)	E1 (Cytosol)	E2 (Class I)	E3 (Class II)	E4 (Mix)
S Mix	21	21	21	21
tRNA	7	7	7	7
P Mix	8.4	8.4	8.4	8.4
Ribosomes	12.6	12.6	12.6	12.6
RNAse Inhibitor	3.5	3.5	3.5	3.5
DNA template	3.23	3.23	3.23	3.23
Mg-Acetate	0	2.8	2.8	2.8
Enzyme	0	3.23	3.23	3.23
PolyP	0	4.2	4.2	4.2
Nuclease-free H ₂ O	12.17	1.94	1.94	1.94
Optiprep	2.1	2.1	2.1	2.1
Total	70 μL	70 μL	70 μL	70 μL

4. RESULTS, OBSERVATIONS, AND CONCLUSIONS

4.1. Assays

Recorded mass of pellets

FTPPK2 Class I pellet: 0.89g

AJPPK2 Class II pellet 0.9g

Joules delivered through sonicator:

FTPPK2 Class I pellet: 220 J

AJPPK2 Class II pellet 208 J

4.1.1. Purification and SDS-PAGE

Conclusion:

As seen in Figure 1, the proteins were pure by SDS-PAGE and EA was used for further steps of the experiment. For both Class I and Class II proteins, each of the proteins fell within range of their expected molecular weight once graphed as seen in Figure 2

4.1.2. Bradford Assay

Deviations:

There were originally two BSA Standards for Class I PPK2. However, the data points for the second BSA standard did not fall in the expected curve and were determined that they should be excluded from the data analysis.

Conclusion:

The class I protein dilutions all fell within ~200 μg/mL, while the class II 1:10 and 1:5 dilutions also fell within ~200 μg/mL, while its furthest data point went up to ~650 μg/mL.

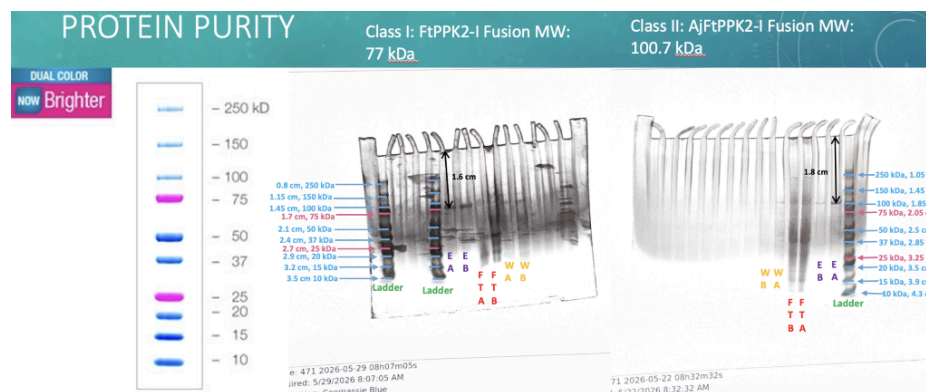


Figure 1. Figure 1. SDS-PAGE stained with Coomassie blue. The ladder band was a mixture of 10 recombinant proteins (10–250 kDa), 8 blue-stained bands, and 2 pink reference bands (25 and 75 kDa). The class I FtPPK2-I Fusion had an expected MW of 77 kDa, and its wells were filled from left to right in the following order: Ladder, Elution A (EA), EB, Flow-through A (FTA), FTB, Wash A (WA), and WB. The class II AjPPK2-I Fusion had an expected MW of 100.7 kDa, and in the image, the wells appear from left to right in the following order: WA, WB, FTA, FTB, EA, EB, and Ladder.

4.1.3. PPK2 activity ASSAYS

Deviations: In the original protocol/lab manual, it calls for a log-log plot. However, we decided to plot

Class II (1/40) Avg		
Time (min)	RLU	[ADP] (M)
0	6.13E+05	6.33E-06
1	9.40E+05	9.60E-06
2	1.27E+06	1.29E-05
4	1.53E+06	1.55E-05
6	1.41E+06	1.43E-05
10	7.65E+05	7.85E-06

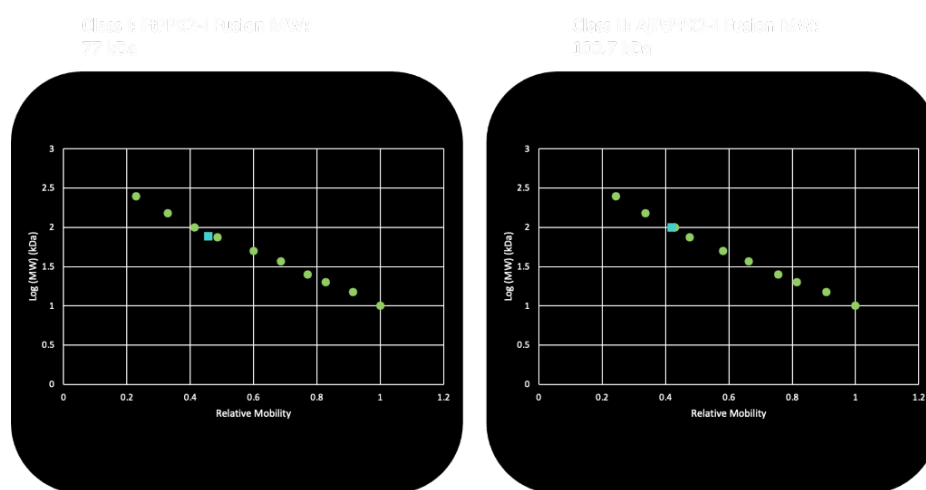


Figure 2. Figure 2. SDS-PAGE Curve of molecular weight and relative mobility of the Standard SDS-PAGE Ladder and the Class I (left figure) and Class II (right figure). Linear relationship present between log molecular weight with respect to distance migrated (measured or relative distances) for the protein standards. These estimates of molecular weights are reasonably accurate, $\pm 5\%$

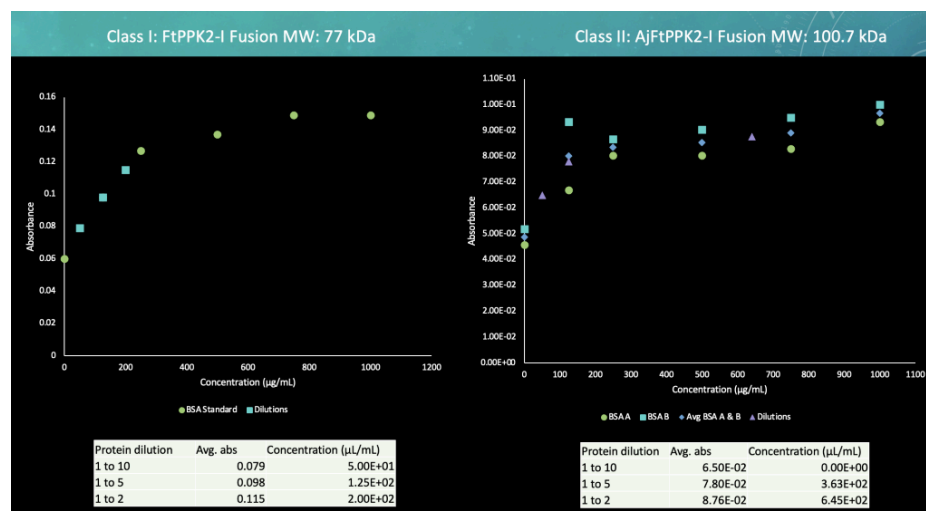
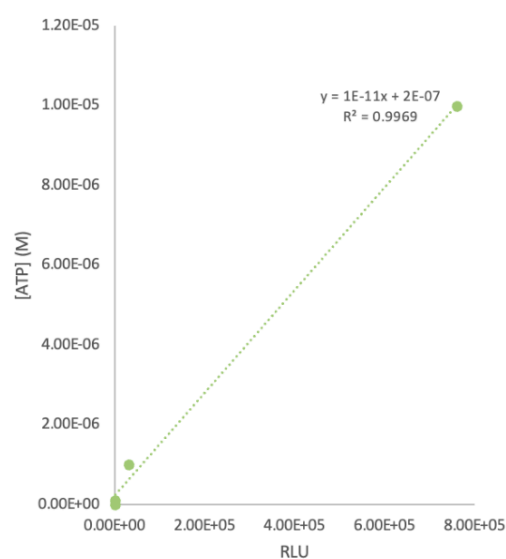


Figure 3. Figure 3. Bradford Assay using Standard BSA concentrations to determine concentrations of the proteins. Each Class had 1:10, 1:5, and 1:2 dilutions created to determine their concentrations with respect to the absorbances. The samples were incubated at room temperature for 5 minutes. They were then read at 595nm for absorbance.

Class I (1/40) Avg		
Time (min)	RLU	[ATP] M
0	5.48E+06	5.50E-05
1	5.47E+06	5.49E-05
2	5.84E+06	5.86E-05
4	3.41E+06	3.43E-05
6	6.57E+06	6.59E-05
10	6.66E+06	6.68E-05

Class I & II (1/40) Avg



ATP Standard Curve	
RLU	[ATP] (M)
1.19E+03	1.00E-08
1.37E+03	1.00E-07
3.10E+04	1.00E-06
7.61E+05	1.00E-05

Figure 4. Figure 4. ATP Standard Curve. Relative luminescence units (RLU) of 10^{-9} , 10^{-8} , 10^{-7} , & 10^{-6} M ATP on a 96-well white opaque plate on the GloMax Discover luminometer at 37°C.

Time (min)	RLU	[ATP] M
0	1.27E+03	2.13E-07
1	1.23E+03	2.12E-07
2	1.11E+03	2.11E-07
4	1.73E+03	2.17E-07
6	1.38E+03	2.14E-07
10	1.31E+03	2.13E-07
Class III (1/40) Avg		
Time (min)	RLU	[ATP] M
1	3.38E+03	4.40E-07
2	3.46E+03	4.52E-07
4	4.69E+03	4.78E-07
6	7.30E+03	5.29E-07
10	1.24E+04	6.36E-07

Table 3. Class I FtPPK2-I ATP, Class II AjPPK2-I ADP, Combination of Class I FtPPK2-I & II AjPPK2-I ATP, and Class III MnPPK2-I ATP time-based assay. Relative luminescence units (RLU) of assay taken on a 96-well white opaque plate on the GloMax Discover luminometer at 30°C over the course of 10 minutes with assays examined at the 0th, 1st, 2nd, 4th, 6th, and 10th minute. ATP/ADP concentrations calculated of the different proteins and their different dilutions using their RLU with the equation on the graph of Figure 4.

Deviation:

The RLU readings of all the different PPK2 activity assays are out of range of the standard ATP curve.

Conclusion:

The concentrations are not accurate and are solely based off the trendline/equation presented by the ATP standard curve (Figure 4).

Next steps: Make a concentration more dilute than 1/40 as the ATP and ADP assays revealed that the dilutions were not dilute enough as they did not fall within the standard curve.

Deviations: Both the 1:40 and 1:10 dilutions were out of range (much higher than) the ATP Standard curve. The class I & class II did not work as predicted as the data points are scattered throughout the graph. The class III data had a curve different than expected, and this data was collected from another group

Conclusion:

The graph of the activity assay of Class II ADP was included to show that it was active and working as expected showing the upwards-plateauing growth of ADP.

AjPPK2 Class II enzyme showed more activity than FtPPK2 Class I, likely because the ADP assay was completed in a smaller period (1 week) of time compared to the ATP Assay (2 weeks), so the enzyme was able to retain a significant amount of activity compared to the ATP Assay. More specifically, for the ATP assay, the assay was run a week before the luminescence was read, whereas the ADP assay was run a day before the luminescence was read.

Overall, the results were inconclusive as to which energy regeneration system worked the best. The reason being that all of the activity readings were done on separate plates and

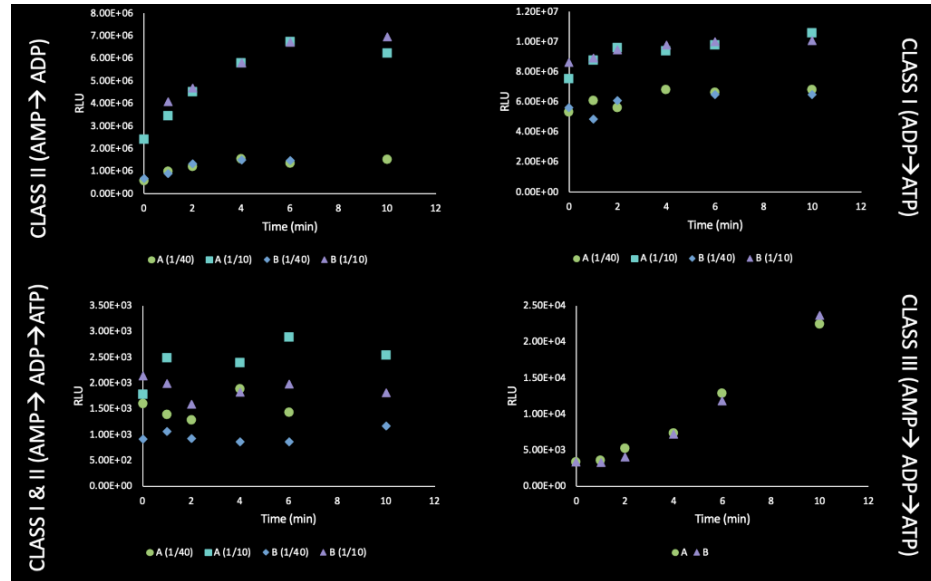


Figure 5. Comparisons of the various PPK2 ATP and ADP production routes. Class I, Class I & II, Class III ATP. Same reaction conditions as described in Figure 4 caption. All graphs contained 1:40 & 1:10 dilutions PPK2, whereas Class III only has a 1:10 dilution (done by Sunayana Nguyen and Emily Cooke)

over the period of a long time causing variation in the experimental environment, causing inconsistencies in relativities throughout the data.

Next steps:

- Incubate Class II with AMP, before combining Class II with Class I. Then, incubate with Class I.
- Testing different combinations of the various Class I and Class II
- Try to do all the experiments in a shorter amount of time so that results are more consistent with each other
- Read all samples and standards on the same plate (the Class III data were read on a separate plate, which prevents direct comparison)
- Have a day to just label centrifuge tubes and prepare dilutions by putting the water into the tubes to maintain same reaction conditions at the very beginning of the experiment, even before purification (very time consuming)

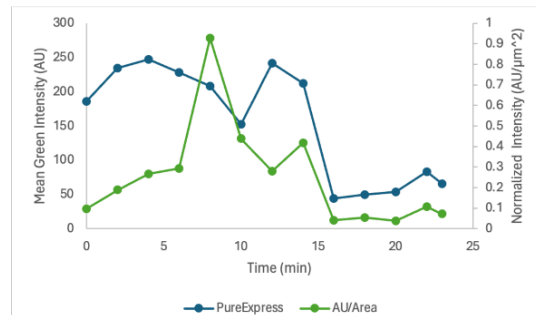
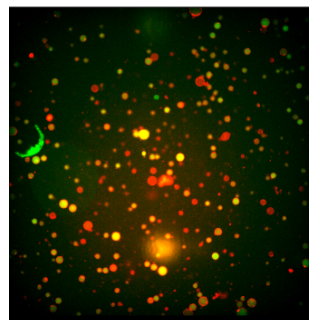


Figure 6. Microscopy screenshot of PURExpress at 0 min (left). Green fluorescence activity of PURExpress encapsulated cells (right). Fluorescence was adjusted to cell area. Average mean green intensity and adjusted intensity values are 154.22 AU and 0.247 respectively.

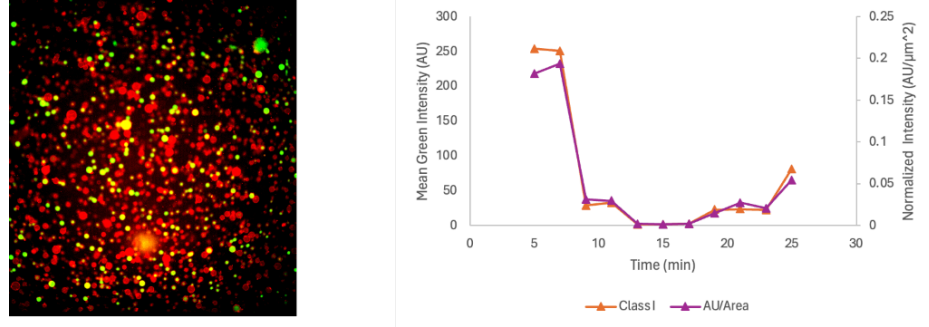


Figure 7. Figure 7. Microscopy screenshot of Class I system at 5 min (left). Green fluorescence activity of class I encapsulated cells (right). Fluorescence was adjusted to cell area. Average mean green intensity and adjusted intensity values are 65.453 AU and 0.051 respectively.

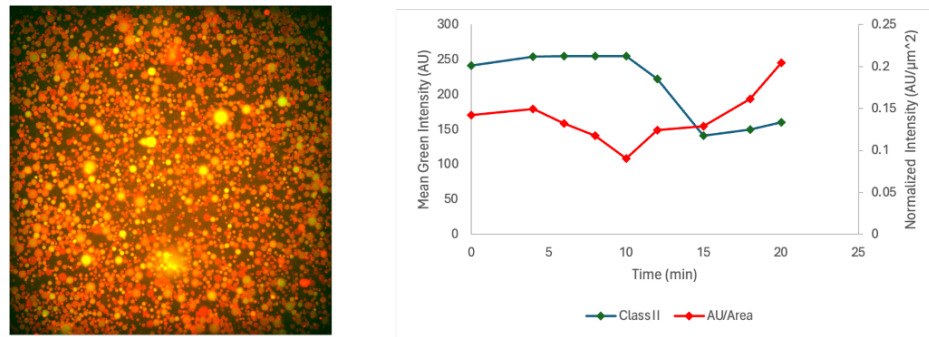


Figure 8. Figure 8. Microscopy screenshot of Class II system at 0 min (left). Green fluorescence activity of class II encapsulated cells (right). Fluorescence was adjusted to cell area. Average mean green intensity and adjusted intensity values are 214.899 AU and 0.139 respectively.

4.2. Encapsulation

4.2.1. PURExpress Encapsulation

4.2.2. Cytosol Encapsulation

*Important note, time stamped microscopy was not performed as only initial encapsulation images were obtained. Therefore, microscopic kinetic profiles are not available.

Figure 12. Microscopy screenshot of Class I system at 0 min. The mean green intensity and adjusted intensity values are 227.036 AU and 1.009 AU/μm² respectively.

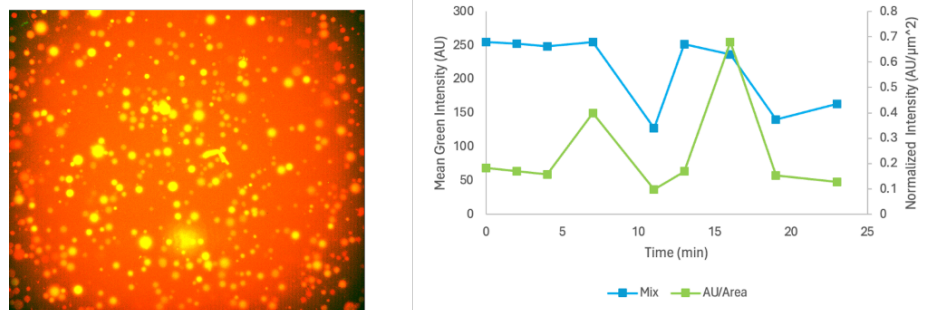


Figure 9. Figure 9. Microscopy screenshot of Class I + II mixture at 0 min (left). Green fluorescence activity of mixture encapsulated cells (right). Fluorescence was adjusted to cell area. Average mean green intensity and adjusted intensity values are 214.291 AU and 0.237 respectively.

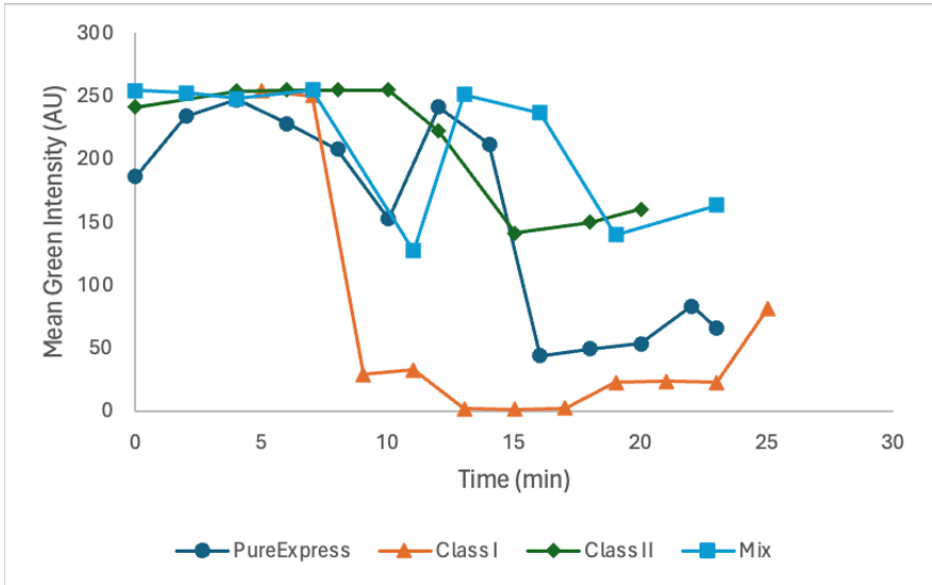


Figure 10. *Figure 10. Green fluorescence activity of encapsulated cells. All four cellular conditions are plotted together.*

Figure 13. *Microscopy screenshot of Class II system at 0 min. The mean green intensity and adjusted intensity values are 249.732 AU and 0.650 AU/μm² respectively.*

Figure 14. *Microscopy screenshot of system mixture at 0 min. The mean green intensity and adjusted intensity values are 179.325 AU and 0.448 AU/μm² respectively.*

4.3. Summary of Results

Table 1. Summary of PURExpress results.

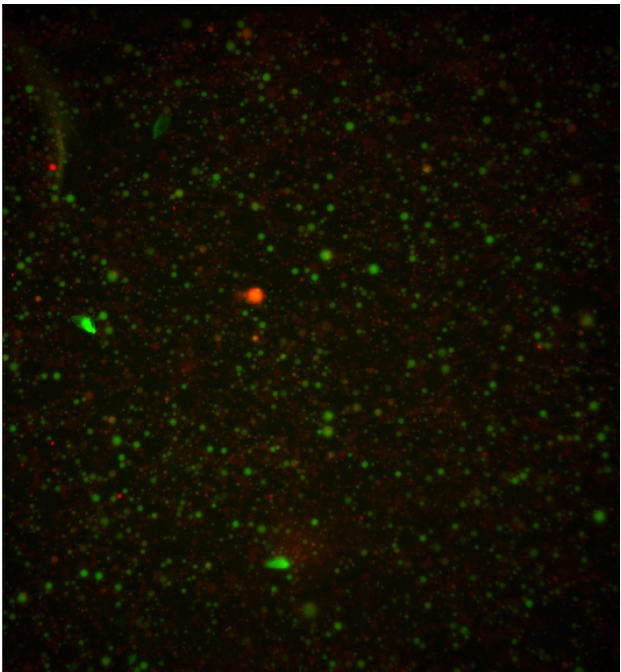


Figure 11. *Figure 11. Microscopy screenshot of Cytosol system at 0 min. The mean green intensity and adjusted intensity values are 114.267 AU and 0.397 AU/μm² respectively.*

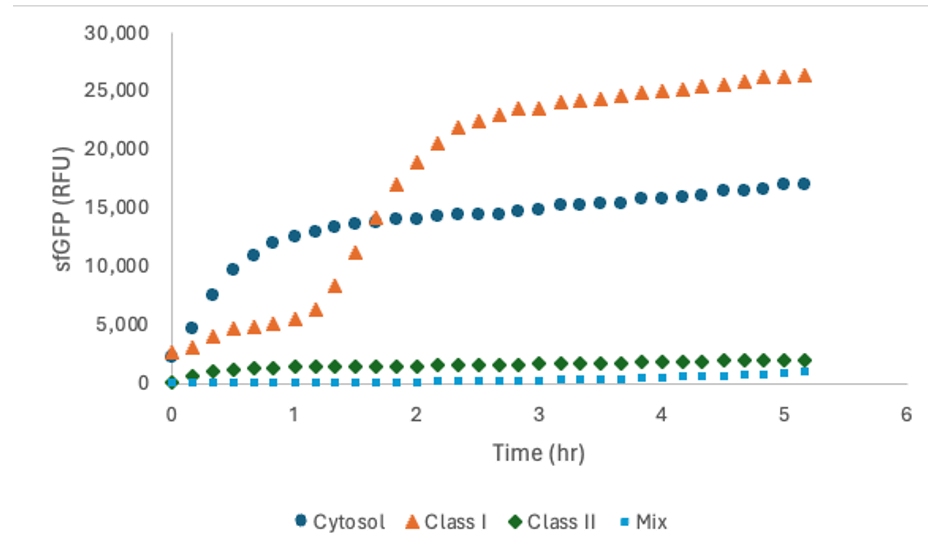


Figure 12. *Figure 15. Protein activity of isolated cytosol cell conditions measured via fluorescence over 5 hour time period. Data represents average of duplicate trials.*

Encapsulation Limitations

There were not any significant problems that occurred regarding the encapsulation procedure. Most limitations were presented during data analysis. In terms of the enzyme efficacy, as mentioned earlier and based on assay results, time was a limiting factor, where it seemed that both enzyme classes eventually expire after purification leading to poor results. Therefore, it is critical that encapsulation occurs without delay.

4.3.1. Quantification Limitations

Upon analyzing the data, there were inherent errors within the ImageJ (Fiji) software, that is not only exclusive to the user. Some images had issues with microscope focus, which influenced the accurate selection and green intensity measurement of liposomes. Sample size was also a problem, where only one cell was selected and measured, which established generalized conclusions for all other cells. Cell movement must also be considered under the microscope, again limiting accurate measurement. Therefore, conclusions had to consider multiple factors and sets of data.

4.3.2. Conclusions

Ultimately, the PURExpress system yielded better results than the cytosol system. Within each respective system, for the PURExpress, the mixture was the most promising, with highest protein translation rates given by the raw mean green and adjusted mean green intensities. The isolated kinetic quantification also confirmed these results with a similar trend. In terms of the cytosol-based system, Class I proved to be most effective, following similar reasoning, but there also is limited data, since microscopic quantification occurred independent of time-dependent fluorescence. Regardless, it appears the mixture was the most effective, supporting initial hypotheses that such a combination could substitute Class III and sufficiently providing exhaustive sources of NMP, NDP, and NTP. Consequently, these cells have extensive supply chains of energy allowing for long lasting protein synthesis and cellular function. Notably, however, inconclusive assay results suggest caution may be necessary before carrying this trend further.