PPK Energy Module in PURExpress

Status	Design
Tags	Developer cells
AI summary	Testing of the PPK energy regeneration module in PURExpress showed that removing creatine phosphate from the energy mix completely inhibited reactions. Subsequent experiments optimized Mg2+ concentration, revealing that the PPK module works best at 16-18 mM Mg2+, and combining it with the CP/CK module significantly increased protein yield.
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Keywords	
Projects	Developer Cell: Energy Module

Overview

The aim is to test the PPK/polyphosphate energy regeneration module in PURExpress.

Background

It has been previously tested by @Surendra Yadav that eliminating just the creatine phosphate substrate from the energy mix for the PURE reactions can completely kill the reactions as there's no substrate available for NTP regeneration. The idea here is to first confirm these observations by removing creatine phosphate from the custom energy mix and using this energy mix for the PURExpress reaction using Solution B. Once, this is established, then the PPK module will be tested.

DREAM

Track experimental progress here. Experiments are not done until written up. Collect results of sequences or series of experiments in

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Close-Out memos and link below.

- [x] ~~Test deletion of creatine phosphate from custom energy mix~~
- [x] ~~Optimise Mg2+ in custom energy mix using creatine phosphate~~
- [x] ~~Test PPK module without creatine phosphate~~
- [x] ~~Optimise Mg2+ in reactions powered by PPK module~~
- [x] ~~Combine PPK and CP/CK module for energy regeneration~~

Notebook

@June 10, 2025

PREPARATION OF CUSTOM ENERGY MIX

3x concentrated Custom Energy Mix was prepared without creatine phosphate. This was used at final 1x concentration in the PURE reactions.

Component	Stock concentration [mM]	Concentration of components in reaction [mM]	Concentration in Energy solution [mM]	Final volume to add [µL]
HEPES	1000	50	150	30.0
Potassium glutamate	2500	100	300	24.0
Magnesium acetate	1000	11.8	35.4	7.1
NTP	100	2	6	12.0
tRNA [mg/mL]	40	3.5	10.5	52.5
Creatine phosphate	1000	0	0	0.0
TCEP	500	1	3	1.2
Folinic acid	5	0.02	0.06	2.4
Spermidine	200	2	6	6.0

Component	Stock concentration [mM]	Concentration of components in reaction [mM]	Concentration in Energy solution [mM]	Final volume to add [µL]
Amino Acid solution	3.25	0.3	0.9	55.4
Water				9.4
Energy solution total		Final concentration [fold]		Final volume
		3		200

PURE REACTION SETUP

The following reactions were setup to test if the deletion of only creatine phosphate can kill the PURE reaction. 50 ul master mix was prepared for each reaction which was then divided into three 15 ul reactions (triplicate) in the 384 well plate.

1. CP/CK Control Reaction with custom energy mix + creatine phosphate

Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
Energy solution-CP	3.00	×	1	×	16.67
Sol B (PURExpress)	3.33	x	1	x	15.00
plam-GFP DNA	120	ng/µL	10	nM	7.96
Creatine phosphate	1000	mM	20	mM	1.00
PEG8K 40%	40	%	2	%	2.50
Water					6.87
Total volume [µL]					
50					
Calculation for DNA concentration	DNA length [bp]	Unit	Avg. MW of bp	Unit	
plam-GFP DNA	2940	bp	650	g/mol	

2. Creatine phosphate deletion from the reaction

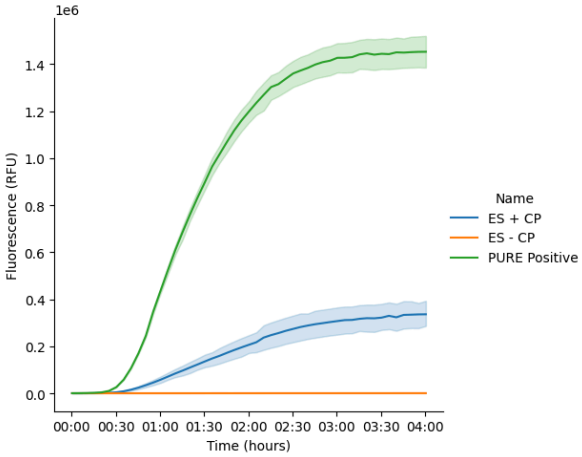
Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
Energy solution-CP	3.00	×	1	×	16.67
Sol B (PURExpress)	3.33	x	1	x	15.00
plam-GFP DNA	120	ng/µL	10	nM	7.96
Creatine phosphate	1000	mM	0	mM	0
PEG8K 40%	40	%	2	%	2.50
Water					7.87
Total volume [µL]					
50					
Calculation for DNA concentration	DNA length [bp]	Unit	Avg. MW of bp	Unit	
plam-GFP DNA	2940	bp	650	g/mol	

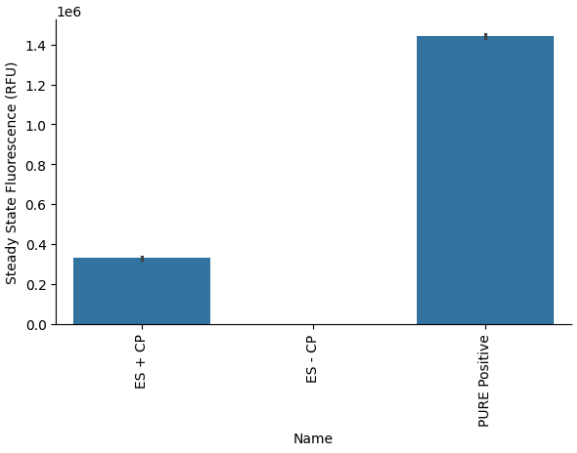
3. PURExpress Positive Control Reaction

Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
Sol A	2.50	×	1	×	20.00
Sol B	3.33	x	1	x	15.00
plam-GFP DNA	120	ng/µL	10	nM	7.96
Water					7.04
Total volume [µL]					
50					
Calculation for DNA concentration	DNA length [bp]	Unit	Avg. MW of bp	Unit	
plam-GFP DNA	2940	bp	650	g/mol	

RESULTS

1. The positive control reaction worked way better than the reaction using the custom energy mix (4x better).
2. The deletion of creatine phosphate completely killed the reaction with negligible protein expression.





CONCLUSION AND FUTURE EXPERIMENT

As the deletion of creatine phosphate kills the reactions, we can now easily test the PPK energy module by replacing creatine phosphate with PPK enzyme and PolyP substrate. Additionally, as the yield was significantly lower for the custom energy mix reaction, it was hypothesised that some Mg2+ optimization might help in achieving higher yields using custom energy mix.

@June 11, 2025

In order to perform Mg2+ optimizations, a new custom energy mix was prepared which didn't have Mg-acetate and creatine phosphate in it. This composition of energy mix is used in all future experiments for this notebook.

PREPARATION OF CUSTOM ENERGY MIX

Component	Stock concentration [mM]	Concentration of components in reaction [mM]	Concentration in Energy solution [mM]	Final volume to add [µL]
HEPES	1000	50	150	30.0
Potassium glutamate	2500	100	300	24.0
Magnesium acetate	1000	11.8	0	0.0
NTP	100	2	6	12.0
tRNA [mg/mL]	40	3.5	10.5	52.5
Creatine phosphate	1000	0	0	0.0
TCEP	500	1	3	1.2
Folinic acid	5	0.02	0.06	2.4
Spermidine	200	2	6	6.0
Amino Acid solution	3.25	0.3	0.9	55.4
Water				16.5
Energy solution total		Final concentration [fold]		Final volume
		3		200

PURE REACTION SETUP

The following reactions were setup to optimise Mg2+ concentration in the PURE reactions utilising custom energy mix. 35 ul master mix was prepared for each reaction which was then divided into three 10 ul reactions (triplicate) in the 384 well plate.

From here onwards, all reactions had 3 nM final DNA concentrations instead of 10 nM to make space in the reaction for other components (if need be).

Final Concentrations of Mg2+ tested: 6, 7, 8, 9, 10, 11, 12 mM

1. Template reaction setup. All reactions had the same composition except the final concentration and volume of Mg2+ added in the reaction.

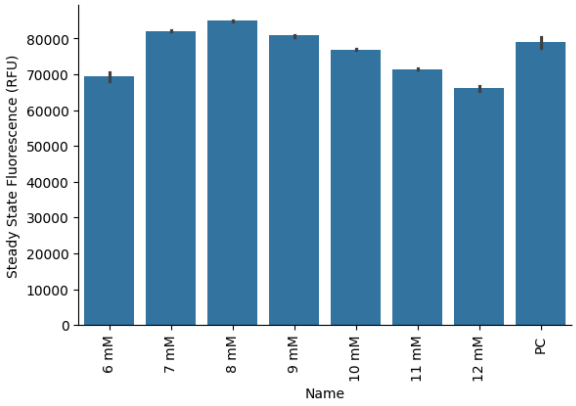
Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
Energy solution-CP	3.00	×	1	×	11.67
Sol B	3.33	x	1.00	x	10.51
plam-GFP DNA	120	ng/µL	3	nM	1.67
Mg-Acetate	200	mM	6	mM	1.05
Creatine phosphate	1000	mM	20	mM	0.70
PEG4K 40%	40	%	2	%	1.75
Water					7.65
Total volume [µL]					
35					
Calculation for DNA concentration	DNA length [bp]	Unit	Avg. MW of bp	Unit	
plam-GFP DNA	2940	bp	650	g/mol	

2. PURExpress Postive Control Reaction

Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
Sol A	2.50	×	1	×	14.00
Sol B	3.33	x	1.00	x	10.51
plam-GFP DNA	120	ng/µL	3	nM	1.67
Water					8.82
Total volume [µL]					
35					
Calculation for DNA concentration	DNA length [bp]	Unit	Avg. MW of bp	Unit	
plam-GFP DNA	2940	bp	650	g/mol	

RESULTS

8 mM Mg2+ concentration provided the highest protein yield, which was comparable to the positive control reaction.



CONCLUSION AND FUTURE EXPERIMENT

Mg2+ concentration was optimised to be 8 mM for the reaction using CP/CK energy module. However, it was observed that 12 mM Mg+ gave higher yields than the ones we got in previous experiment where Mg2+ concentration was 11.8 mM. This difference can probably be attributed to direct addition of Mg2+ in this reaction, whereas previously Mg2+ was added in the energy mix which could result in chelation of a fraction of Mg2+ by the amino acids and NTPs in the energy mix. However, this is just a hypothesis and not a conclusion, further experiments are required to verify this effect.

@June 12, 2025

After Mg2+ optimisation for CP/CK module, PPK module was implemented in PURE by replacing the creatine phosphate with PPK and polyP substrate. As the Mg2+ concentration is crucial for different energy modules, we tested the PPK module with a varying ranging of final Mg2+ concentrations in the reaction (8, 10, 12, 14 mM).

Additionally, to assess the effect of polyP addition on a normal PURE reaction, a control CP/CK + polyP reaction was setup.

PURE REACTION SETUP

1. Template reaction setup. All reactions had the same composition except the final concentration and volume of Mg2+ added in the reaction.

Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
Energy solution-CP	3.00	×	1	×	11.67
Sol B	3.33	x	1.00	x	10.51
plam-GFP DNA	120	ng/µL	3	nM	1.67
Mg-Acetate	200	mM	8	mM	1.40
Creatine phosphate	1000	mM	0	mM	0.00
PEG4K 40%	40	%	2	%	1.75
PolyP	500	mM	30	mm	2.10
PPK2	57.5	uM	2	uM	1.22
Water					4.68
Total volume [µL]					
35					
Calculation for DNA concentration	DNA length [bp]	Unit	Avg. MW of bp	Unit	
plam-GFP DNA	2940	bp	650	g/mol	

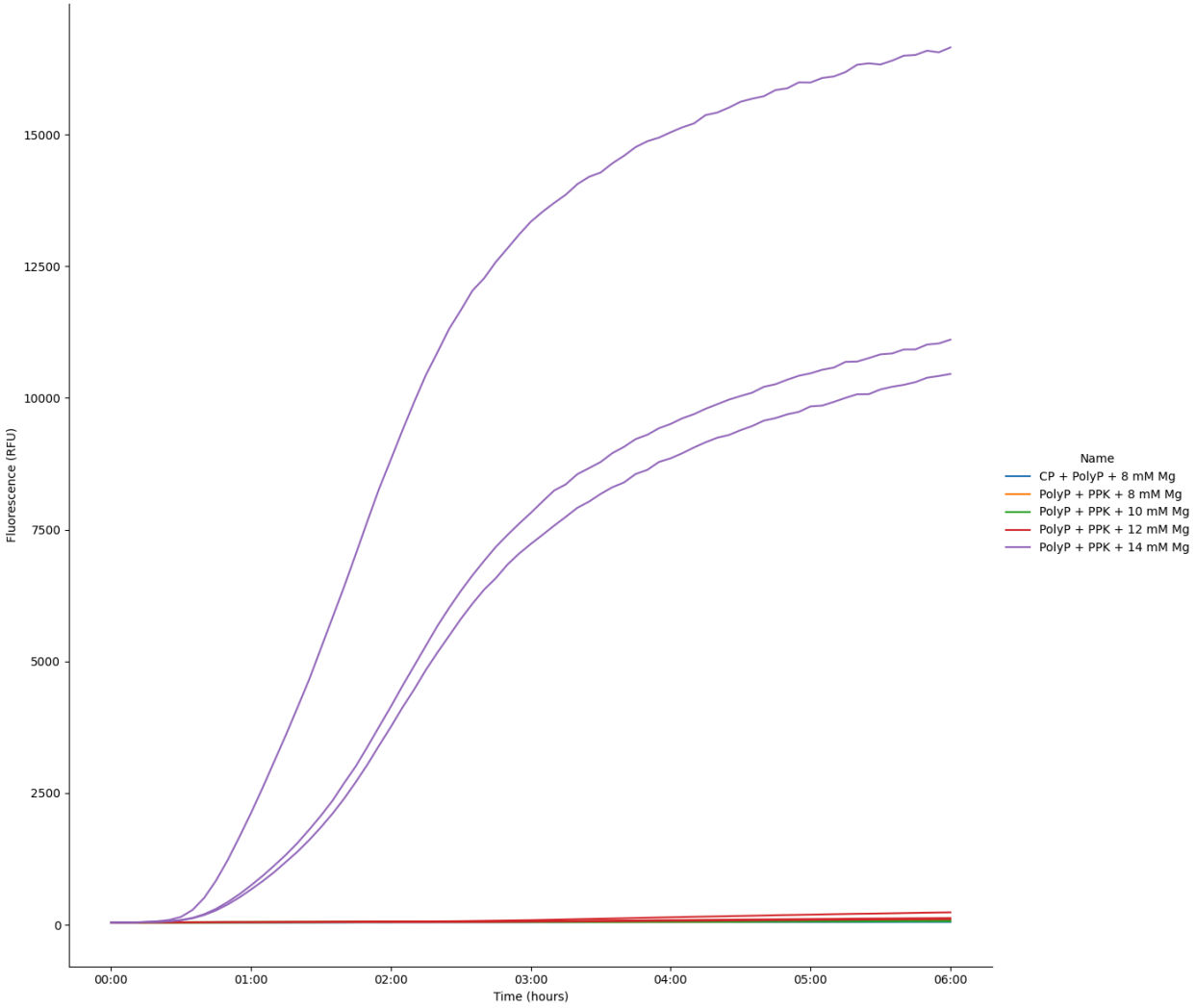
2. CP/CK + Polyp Control

Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
Energy solution-CP	3.00	×	1	×	11.67
Sol B	3.33	x	1.00	x	10.51
plam-GFP DNA	120	ng/µL	3	nM	1.67
Mg-Acetate	200	mM	8	mM	1.40
Creatine phosphate	1000	mM	20	mM	0.70
PEG4K 40%	40	%	2	%	1.75
PolyP	500	mM	30	mm	2.10
Water					5.20

Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
Total volume [µL]					
35					
Calculation for DNA concentration	DNA length [bp]	Unit	Avg. MW of bp	Unit	
mplam-GFP DNA	2940	bp	650	g/mol	

RESULTS

1. PPK Module reactions worked only with higher concentration of Mg2+ at 14 mM.
2. Addition of polyP inhibited the control CP/CK reaction at 8 mM Mg2+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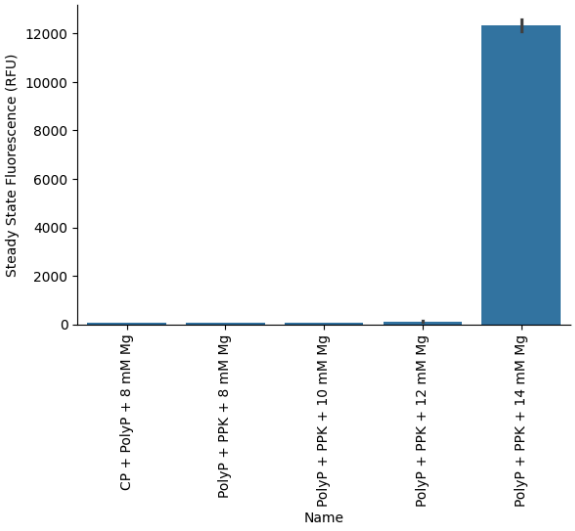
PolyP + PPK + 12 mM Mg
PolyP + PPK + 14 mM Mg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PolyP + PPK + 12 mM Mg
PolyP + PPK + 14 mM Mg
Name

CONCLUSION AND FUTURE EXPERIMENT

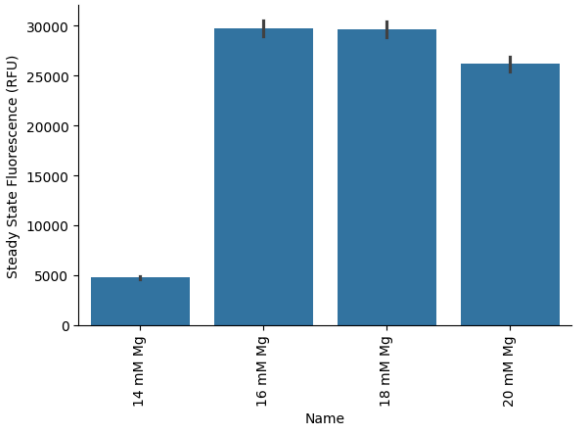
A conclusive take away from this experiment is that the PPK module works in the PURE reaction at 14 mm Mg2+. In order to further optimise Mg2+ concentrations in PURE using PPK module, we might need to add even higher concentration of Mg2+ (>14 mM).

@June 13, 2025

To further optimise Mg2+ concentrations in PPK module reactions, we tested even higher concentrations of Mg2+. Everything else remained the same as in previous experiment except the final Mg2+ concentrations in reactions.

RESULTS

1. Highest protein yields were obtained using 16, and 18 mM Mg2+, whereas protein yield showed a decline with 20 mM concentration.
2. Just to point it out, the results of 14 mM Mg2+ were not the same as obtained in previous experiment. The final yield seems to have gone down to around 5000 rfu from 12000 rfu. The reason for this can be either pipetting error or using a different stock of Mg2+ solution.



CONCLUSION AND FUTURE EXPERIMENT

Conclusion: PPK module works as energy regeneration component in the PURE system, performing optimal at 16-18 mM Mg+ concentration. However, the yield can be improved with substrate (polyP) and enzyme (PPK) optimisation.

The next idea was to combine both the CP/CK and the PPK module in the PURE reaction for optimum protein expression and yield.

@June 16, 2025

To combine the PPK and CP/CK Energy modules in PURExpress, the reactions were supplemented with creatine phosphate, polyP, and PPK enzyme. Composition of different reactions is described below.

REACTION SETUP

1. PURE with CP and 8 mM Mg2+ (Mg optimised on 11 June for CP/CK)

Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
Energy solution-CP	3.00	×	1	×	11.67
Sol B	3.33	x	1.00	x	10.51
plam-GFP DNA	120	ng/µL	3	nM	1.67
Mg-Acetate	200	mM	8	mM	1.40
Creatine phosphate	1000	mM	20	mM	0.70
PEG4K 40%	40	%	2	%	1.75
PolyP	500	mM	0	mm	0.00
PPK2	57.5	uM	0	uM	0.00
Water					7.30
Total volume [µL]					
35					
Calculation for DNA concentration	DNA length [bp]	Unit	Avg. MW of bp	Unit	
plam-GFP DNA	2940	bp	650	g/mol	

2. PURE with PolyP, PPK and 18 mM Mg2+

Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
Energy solution-CP	3.00	×	1	×	11.67
Sol B	3.33	x	1.00	x	10.51
plam-GFP DNA	120	ng/µL	3	nM	1.67
Mg-Acetate	200	mM	18	mM	3.15
Creatine phosphate	1000	mM	0	mM	0.00
PEG4K 40%	40	%	2	%	1.75
PolyP	500	mM	30	mm	2.10
PPK2	57.5	uM	2	uM	1.22
Water					2.93
Total volume [µL]					
35					
Calculation for DNA concentration	DNA length [bp]	Unit	Avg. MW of bp	Unit	
plam-GFP DNA	2940	bp	650	g/mol	

3. PURE with CP, PolyP, PPK and 18 mM Mg2+

Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
Energy solution-CP	3.00	×	1	×	11.67
Sol B	3.33	x	1.00	x	10.51
plam-GFP DNA	120	ng/µL	3	nM	1.67
Mg-Acetate	200	mM	18	mM	3.15
Creatine phosphate	1000	mM	20	mM	0.70
PEG4K 40%	40	%	2	%	1.75
PolyP	500	mM	30	mm	2.10
PPK2	57.5	uM	2	uM	1.22
Water					2.23
Total volume [µL]					

Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
35					
Calculation for DNA concentration	DNA length [bp]	Unit	Avg. MW of bp	Unit	
plam-GFP DNA	2940	bp	650	g/mol	

4. PURExpress Positive control (PC) reaction

Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
Sol A	2.50	×	1	×	14.00
Sol B	3.33	x	1.00	x	10.51
plam-GFP DNA	120	ng/µL	3	nM	1.67
Mg-Acetate	200	mM	0	mM	0.00
Creatine phosphate	1000	mM	0	mM	0.00
PEG4K 40%	40	%	0	%	0.00
PolyP	500	mM	0	mm	0.00
PPK2	57.5	uM	0	uM	0.00
Water					8.82
Total volume [µL]					
35					
Calculation for DNA concentration	DNA length [bp]	Unit	Avg. MW of bp	Unit	
plam-GFP DNA	2940	bp	650	g/mol	

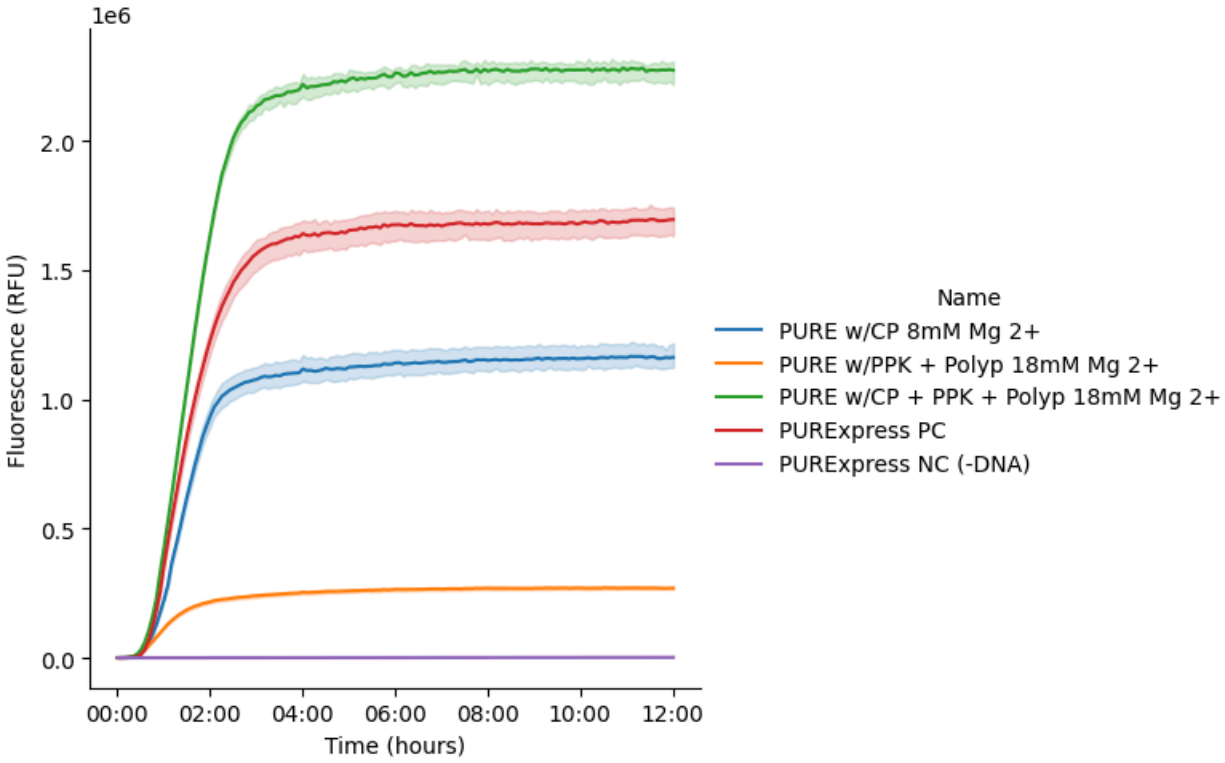
5. PURExpress Negative control (NC) reaction (without DNA template)

Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
Sol A	2.50	×	1	×	14.00
Sol B	3.33	x	1.00	x	10.51
plam-GFP DNA	120	ng/µL	0	nM	0.00
Mg-Acetate	200	mM	0	mM	0.00

Component	Input concentration	Unit	Final concentration	Unit	Volume for one reaction [µL]
Creatine phosphate	1000	mM	0	mM	0.00
PEG4K 40%	40	%	0	%	0.00
PolyP	500	mM	0	mm	0.00
PPK2	57.5	uM	0	uM	0.00
Water					10.49
Total volume [µL]					
35					
Calculation for DNA concentration	DNA length [bp]	Unit	Avg. MW of bp	Unit	
plam-GFP DNA	2940	bp	650	g/mol	

RESULTS

Combining CP/CK and PPK energy regeneration modules provided much higher yield than the reactions powered either by CP/CK or PPK module alone.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PURE w/PPK + Polyp 18mM Mg 2+
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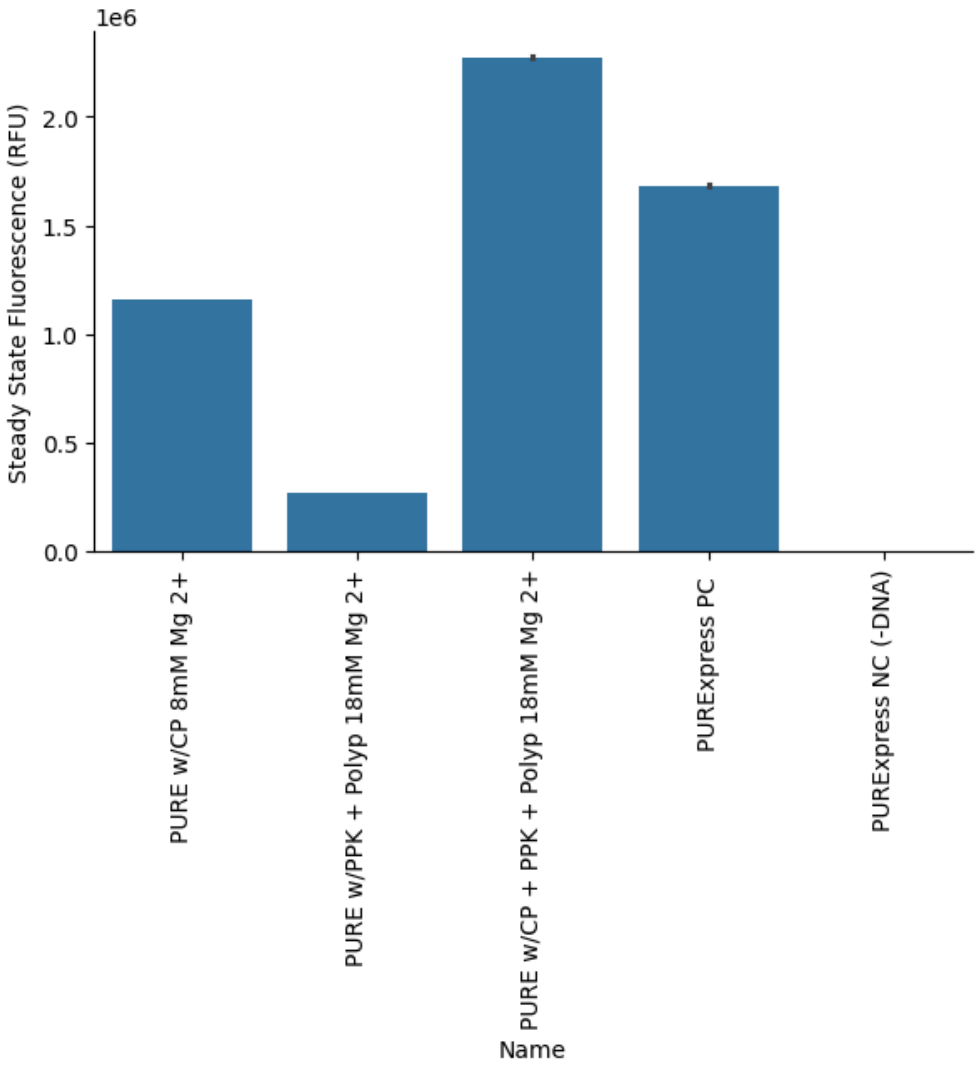
PURExpress PC
PURExpress NC (-DNA)



CONCLUSION

Through these set of experiments we have established that PPK module can be implemented in the PURE system as an energy regeneration module and additionally, combining CP/CK and PPK modules provides increased protein yield than the reactions powered either by CP/CK or PPK module alone.

The experiments below are done by @Yen-Yu Hsu

@June 30, 2025

To repeat the previous experiment but replacing PEG4K with Optiprep.