Supporting Information

Genetically expanded cell free protein synthesis using endogenous pyrrolysyl orthogonal translation system.

Yonatan Chemla[1], Eden Ozer[1], Orr Schlesinger[1], Vincent Noireaux[2], Lital Alfonta[1]*

[1]Department of Life Sciences and Ilse Katz Institute for Nanoscale Science and Technology, Ben-Gurion University of the Negev, P.O. Box 653, Beer-Sheva, 8410500,Israel.

[2]School of Physics and Astronomy, University of Minnesota, Minneapolis, Minnesota 55401, USA

*Corresponding Author: Lital Alfonta: alfontal@bgu.ac.il;

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Table S1. Different transformations performed in each experiment conducted in this study

Plasmid / Strain	pEVOL PylRS (Pyl-OTS)[a]	pEVOL PylRS-AF (PylAF-OTS)[b]	pSUP pACF (pAcF-OTS)[c]	pKD SepRS (Sep-OTS)[d]	No OTS
C321.Δ*prf*A	+	+	+	+	+
C321.Δ*prf*A EXP	+				
C321 (i.e. *prf*A+)	+				
BL21(DE3)	+				
DH5α	+				

[a] Pyl-OTS: Mm-pyrrolysil synthetase/Mm-tRNA$_{CUA}^{Pyl}$ (Blight et al., 2004; Srinivasan et al., 2002) [b] PylAF-OTS: Mm-pyrrolysil synthetase/Mm-tRNA$_{CUA}^{Pyl}$ (Herner et al., 2013). [c] pAcF-OTS: Mj-para-aceto-phenylalanyl synthetase/Mj-tRNA$_{CUA}^{Opt}$ (Wang et al., 2003). [d]Sep-OTS: Mm-phospho-seryl synthetase/Mm-tRNA$_{CUA}^{Sep}$ and Ef-sep (an orthogonal elongation factor) (Park et al., 2011).

Table S2. Strains that were used for CFPS extract preparation and plasmids that were used both as OTS in the Extract strains and as expression template for the CFPS reactions.

Strains/ Plasmids	Details, Use & Rational of use	References
Strains		
C321.ΔprfA	Genomically recoded *E. coli* having all (321) TAG nonsense codons replaced and release factor 1 (RF1) knockout, making it ideal for Amber suppression (genetic code expansion). Herein, because of its attributes used as main chassis. (Cm^R)	(Lajoie et al., 2013) , (Addgene #48998)
C321 (RF1+)	Same as above, release factor 1 has not been deleted. Used as a control for the effect of RF1 on suppression efficiency. (Cm^R)	(Lajoie et al., 2013), (Addgene #48999)
DH5α	*(F- endA1, glnV44, thi-1, recA1, relA1, gyrA96, deoR, nupG, Φ80dlacZΔM15, Δ(lacZYA-argF)U169, hsdR17(rK-mK+), E. coli* strain that transforms with high efficiency. Like many cloning strains, DH5 alpha has several features that make it useful for recombinant DNA methods.	(Phue et al., 2008; Taylor et al., 1993) (NEB product #C2987H)
BL21(DE3)	*(F_, ompT, hsdSB (rB_,mB), dcm, gal) E. coli* strain that express proteins (Also under T7 promoters) and replicates plasmid DNA with high efficiency.	(Phue et al., 2008)
Plasmids		
pEVOL *Mm*PylRS/*Mm*PyltRNA (i.e. the Pyl-OTS)	Orthogonal translation system (OTS) plasmid containing 2 copies of the *MmPylRS* gene under the regulation of araBAD and the glns promoters (induced by arabinose) and the *MmPylT* gene under the regulation of the proK promoter. (Cm^R)	(Young et al., 2010) (Kind gift from E. Lemke)
pEVOL *Mm*PylRS-AF/*Mm*PyltRNA	Same as above but the *MmPylRS* gene mutated to accept 1,3-benzothiazole (bioorthogonal fluorescent dyes) derivatives (Cm^R)	(Herner et al., 2013) (Kind gift from Prof. E. Lemke)
pSUP pAcF	Orthogonal translation system (OTS) plasmid containing the MjTyrRS (pACF) gene under the regulation of glnS promoter and 6 copies of the MjTyrT genes under the regulation of 2 different proK promoters. (Cm^R)	(Ryu and Schultz, 2006) (Kind gift from P. G. Schultz).
pKD SepRS, EFSep , 5x	Orthogonal translation system (OTS) used in	(Park et al.,

tRNASep (B40 OTS)	phosphoprotein synthesis. Expresses the *MjSep-accepting tRNA (tRNASep),* the *M. Maripaludis Sep-tRNA synthetase (SepRS)* and an engineered EF-Tu (EFSep) (Kan^R)	2011) (Addgene #52054)
pBEST-OR2-OR1-Pr-UTR1-deGFP-T500	Expression plasmid, *deGFP* expression is regulated by the OR2-OR1 promoter (bacteriophage Lambda promoter with one mutation). The *deGFP* gene was mutated to create the following variants: Y35X (i.e. Y35TAG mutation) (Amp^R)	(Sun et al., 2013) (Addgene #40019)

Table S3. A comparison between genetic code system/methodologies

Item	Endogenous Code expanding CFPS	Exogenous code expanding CFPS	In vivo
Time from PCR product to protein expression	Reaction: Overnight	tRNA synthesis : 2 days aaRS synthesis : 2 days Reaction: Overnight	Co-Transformation: 1day Expression: 1day Purification: 1day
Expression vector needed	No	No	Yes
Amount of UAA needed	~1 μmol(UAA)/mg(Protein)	~1 μmol(UAA)/mg(Protein)	~100 μmol(UAA)/mg(Protein)
Storage time	<1 year	o-tRNA : ~1.5 month o-aaRS: ~ 2-4 weeks	N/A
o-tRNA maturation and nativity	Complete	Either synthetic or cell based but purified using organic solvents.	Complete
aaRS folding and nativity	Complete	Purification tags and processes needed	Complete
Use of insoluble molecules (PylRS derivatives)	Possible	Not reported	Possible
Reaction preparation time	~30 minutes	~1 hour	NA
No. of different component/processes (Levels of complexity)	3 (DNA, lysate and reaction buffer)	5 (DNA, lysate, Buffer, tRNA and aaRS). Last two items could go wrong both upstream and in downstream applications.	NA
Downstream processes of product	Instantly ready for immune assays, protein assays, chemical reactions, calorimetric assays and purification.		Needs to undergo lysis before ready for downstream.
Tracking kinetics of protein expression	Using fluorescent tags or calorimetric assays enable live tracking of expression kinetics		No kinetic tracking.
Reproducibility of results	High	Medium	High
Absolute protein yields	> 1 mg	> 1 mg	< 1 mg
Relative protein yields	~1mg/ml(reaction)	~1mg/ml(reaction)	0.02mg/ml(culture)
Scale-up	Complex – But have 2 components less to independently produce	Complex	Easy

	and scale up		
Simultaneous reactions with different DNA templates	Limitless, can easily create arrays.		Limited – transformation, growth and sorting are needed.

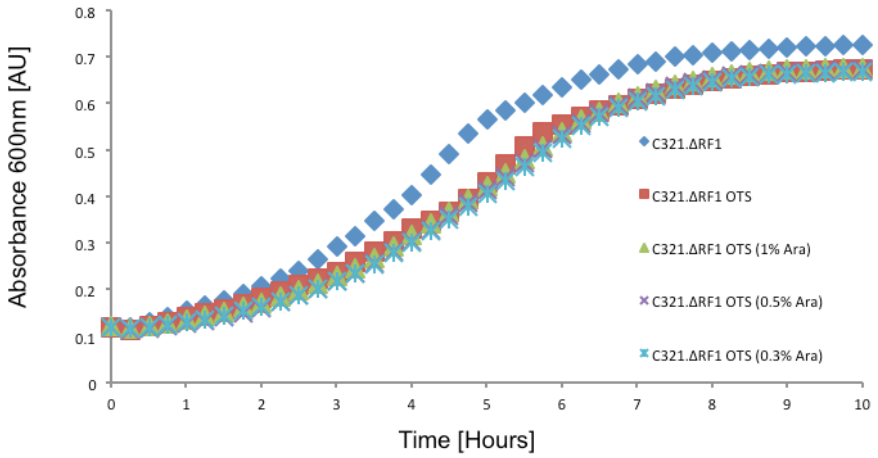


Figure S1. Growth curves of the C321.Δ*prf*A strain and C321.Δ*prf*A cells transformed to express Pyl-OTS from plasmid pEVOL MmPylRS/MmPyltRNA ,using varying arabinose concentrations. Each data point on the graph represents 10 sample repeats.

From the results, it is clear that the growth curves of all OTS-transformed bacteria are close to identical under all inducer concentrations tested, and they grow at a rate similar to the parent GRO strain. The small difference in the growth between the transformed and parent strains could be explained by the energy/resources spent by the transformed bacteria to over-express the plasmid genes and the need to replicate those genes in high copy numbers. These results led us to conclude that no noticeable toxicity was caused by introduction of the Pyl-OTS into the GRO parental strain. This may be explained by the fact that the GRO strain, having all of its TAG codons removed, does not suffer from toxicity from due to read-through of any amber suppressors.

Growth measurements method. C321.Δ*prf*A and C321.Δ*prf*A pEVOL PylRS strains were incubated overnight in LB broth at 30°C and 275 rpm. Both cultures were then diluted 1/100 to OD_{600} of ~ 0.1. The C321.Δ*prf*A pEVOL PylRS were then divided into 4 cultures each being inducted with different L-Arabinose (Chem-Impex Int'l Inc. , wood dale, IL) concentration; 0% (Not induced), 0.3% Arabinose, 0.5% Arabinose and 1% Arabinose. 100 µL of each diluted culture were added to Nunclon Delta Surface 96 well plate (Thermo Fisher Scientific Nunc, Roskilde, Denmark). OD_{600} was measured every 15 min while incubating at 30°C with fast shaking in Synergy HT plate reader (Biotek, Winooski, VT). Data was collected during 15 h from 10 repeats of each culture.

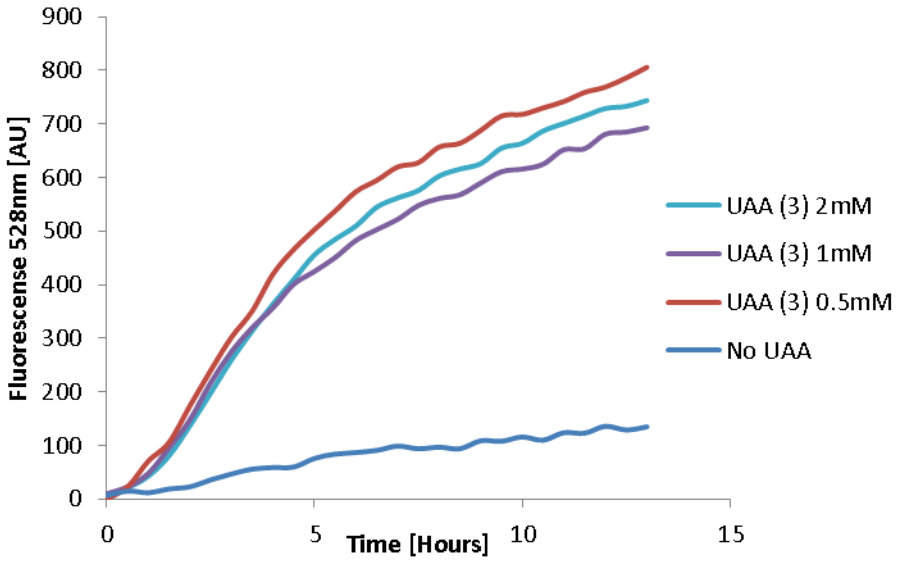
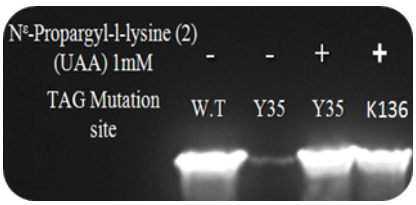


Figure S2. N^{ε}-Boc-L-lysine Incorporation into deGFP while testing various UAA (**3**) concentrations as can be seen at concentrations between 1-2 mM of (3) the reaction reaches similar values.

A)



B)

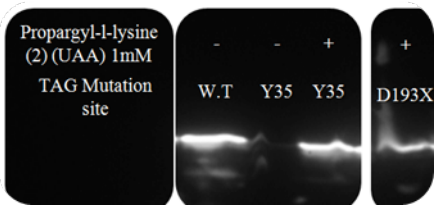


Figure S3. Successful incorporation N^{ε}-Propargyl-L-lysine in K136X (Panel A) and D193X (Panel B) using the endogenous and genetically expanded CFPS system, with the Pyl-OTS.

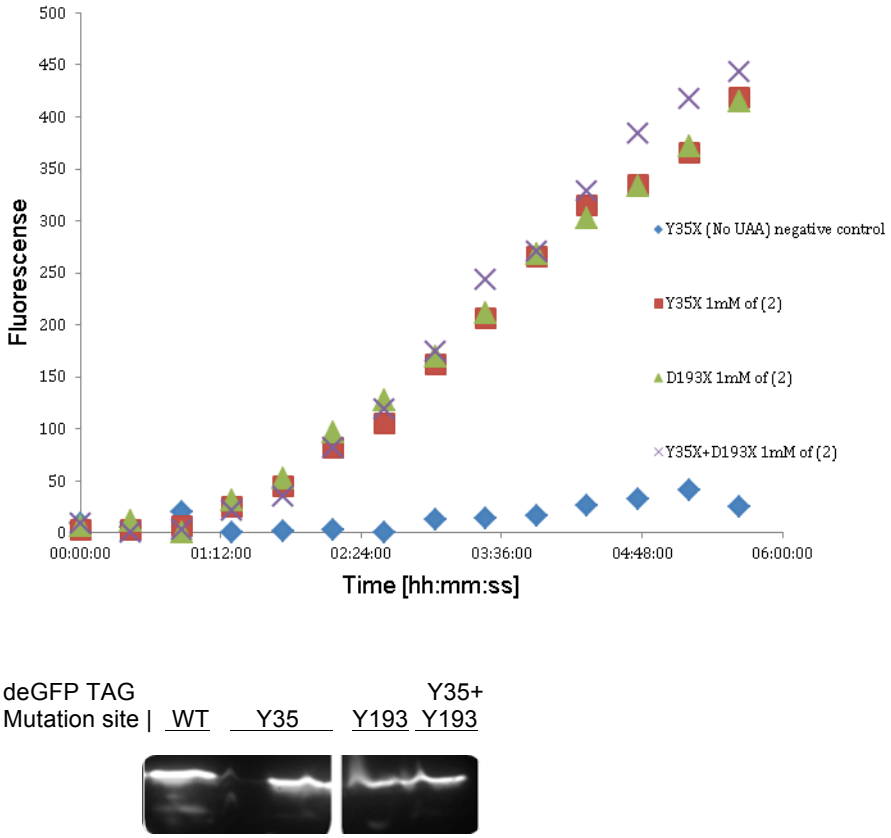
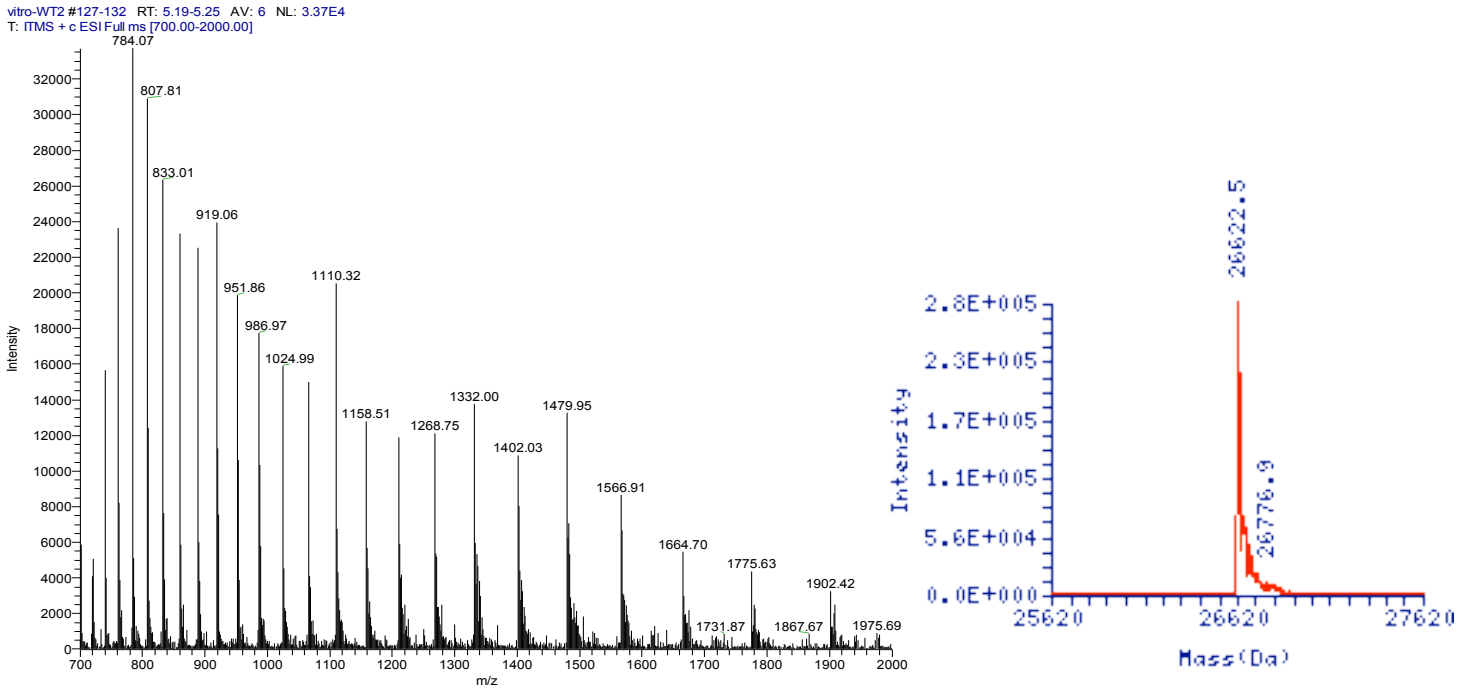


Figure S4. Upper panel; kinetics of N^{ε}-Propargyl-L-lysine (**2**) incorporation in multiple sites of deGFP while using the endogenous and genetically expanded, with the Pyl-OTS. Bottom panel: Anti GFP western blotting of the genetically expanded CFPS showing the successful incorporation of (**2**) in the each of the two sites individually and combined.

Figure S5. a) Detailed LC\MS data for WT deGFP (6xhis)



Mass Peak List Sorted by Intensity:

Mass(Da)	+- Std. Dev.	Intensity	Score	Delta Mass	%Relative	%Total
26622.5	3.1	2.82E+005	77.61	0.0	100.0	42.3
26611.5	1.9	8.32E+004	2.33	-11.0	29.5	12.5
26657.8	6.8	7.15E+004	61.55	35.3	25.4	10.7
26641.1	1.8	5.21E+004	32.89	18.6	18.5	7.8
26687.0	5.1	4.60E+004	55.40	64.5	16.3	6.9
26671.8	1.6	3.17E+004	11.12	49.3	11.2	4.8
26706.6	3.0	2.01E+004	36.20	84.1	7.1	3.0
26725.4	6.7	1.96E+004	46.12	102.9	7.0	2.9
26776.9	4.9	1.01E+004	13.86	154.4	3.6	1.5
26801.3	6.1	9.64E+003	36.29	178.8	3.4	1.4
26742.2	3.1	8.12E+003	12.03	119.7	2.9	1.2
26756.8	4.0	7.98E+003	15.07	134.3	2.8	1.2
26818.5	2.3	7.30E+003	15.25	196.0	2.6	1.1
26790.9	1.5	6.41E+003	3.27	168.4	2.3	1.0
26838.2	3.7	6.30E+003	11.90	215.7	2.2	0.9
26861.6	3.8	2.48E+003	6.75	239.1	0.9	0.4
26894.5	6.3	1.62E+003	2.08	272.0	0.6	0.2

Coomassie stained SDS PAGE gel showing the purification results of the WT deGFP prior to LC/MS.

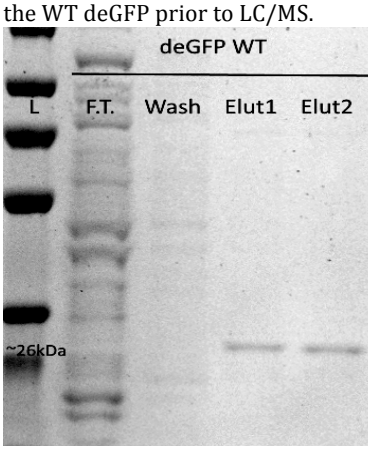
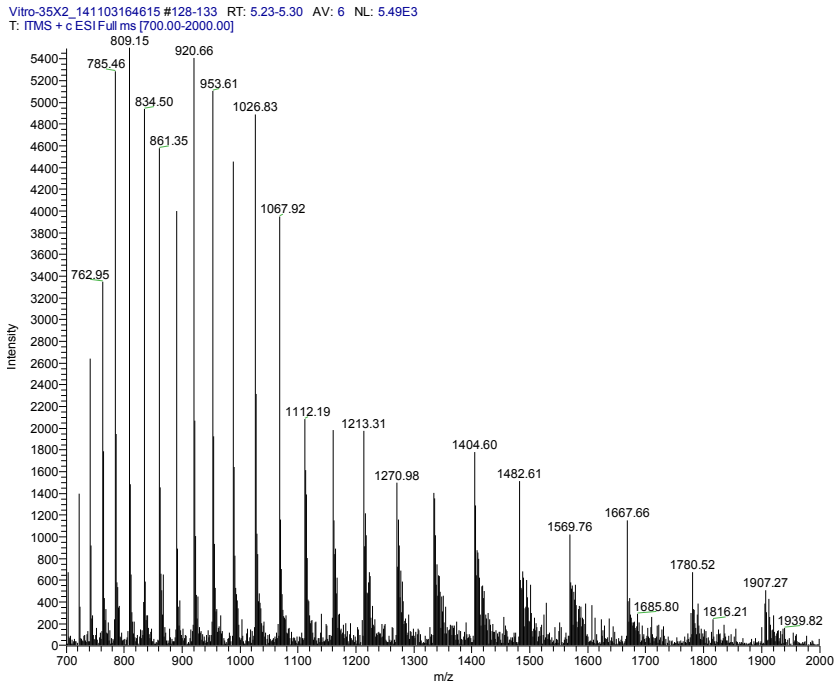
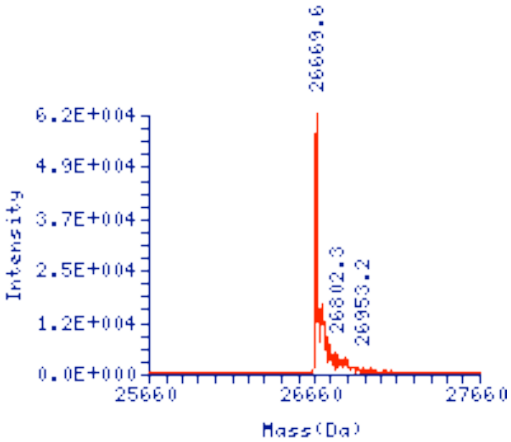


Figure S6. b) Detailed LC\MS data for Y35**(2)** deGFP (6xhis)

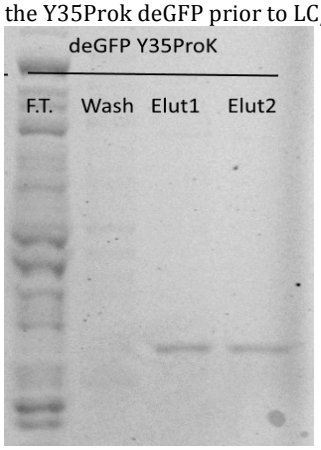




Mass Peak List Sorted by Intensity:

Mass(Da)	+- Std. Dev.	Intensity	Score	Delta Mass	%Relative	%Total
26669.6	2.2	6.16E+004	73.90	0.0	100.0	40.9
26701.4	5.9	1.63E+004	51.95	31.8	26.5	10.8
26714.5	1.6	1.19E+004	8.57	44.9	19.3	7.9
26686.9	1.6	9.69E+003	14.95	17.3	15.7	6.4
26728.2	5.5	8.45E+003	49.26	58.6	13.7	5.6
26753.8	2.9	6.43E+003	42.73	84.2	10.4	4.3
26739.6	1.7	6.12E+003	5.31	70.0	9.9	4.1
26774.1	8.9	5.00E+003	42.47	104.5	8.1	3.3
26802.3	4.7	4.25E+003	38.09	132.7	6.9	2.8
26850.8	5.6	3.52E+003	36.58	181.2	5.7	2.3
26822.6	4.2	2.92E+003	23.81	153.0	4.7	1.9
26787.4	2.1	2.86E+003	5.38	117.8	4.6	1.9
26837.2	1.5	2.08E+003	8.65	167.6	3.4	1.4
26890.8	6.0	1.51E+003	13.96	221.2	2.4	1.0
26870.6	3.4	1.47E+003	7.02	201.0	2.4	1.0
26921.1	5.1	1.36E+003	7.92	251.5	2.2	0.9
26953.2	2.9	1.24E+003	6.13	283.6	2.0	0.8
27002.3	4.8	1.16E+003	3.70	332.7	1.9	0.8
26976.3	6.6	6.19E+002	3.50	306.7	1.0	0.4
27126.7	5.2	5.40E+002	2.12	457.1	0.9	0.4
27022.9	6.2	5.10E+002	3.89	353.3	0.8	0.3
24190.1	5.2	4.65E+002	2.06	-2479.5	0.8	0.3
22789.0	3.4	3.95E+002	2.58	-3880.6	0.6	0.3
27079.1	7.4	3.62E+002	2.06	409.5	0.6	0.2

Coomassie stained SDS PAGE gel showing the purification results of the Y35Prok deGFP prior to LC/MS.



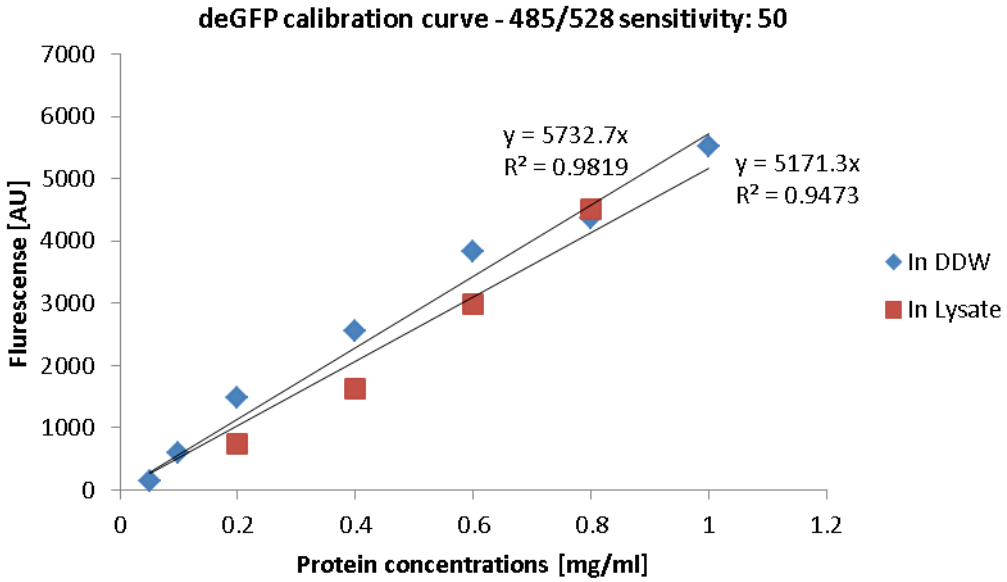


Figure S7. Calibration curve for correlating between EGFP florescence and protein concentration.

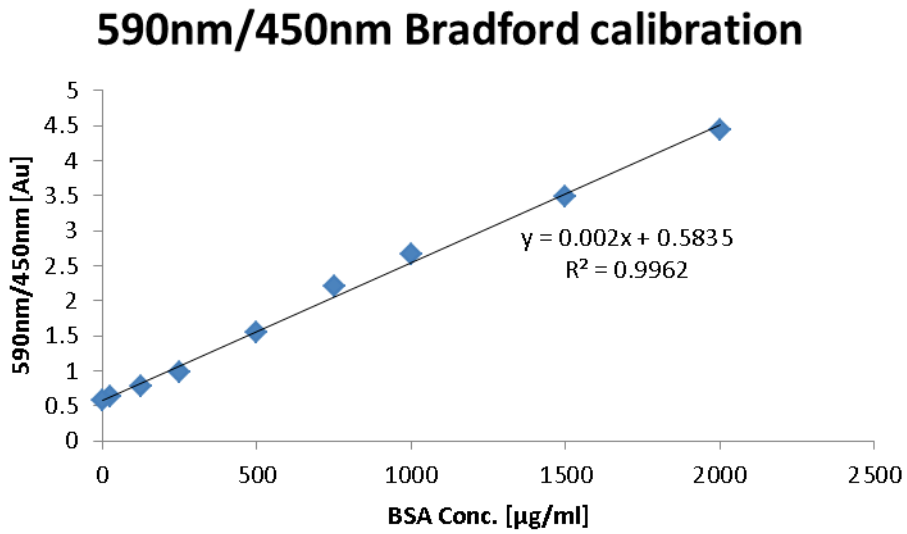


Figure S8 - Bradford Calibration Curve**.**

The Bradford assay results:

Bradford Assay Data	WT deGFP 1	WT deGFP 2	Y35**(2)** deGFP 1	Y35**(2)** deGFP 2
590nm/450nm	0.89	0.88	0.99	1.01
Protein concentration [ug/ml]	152.12	145.75	204.88	215.08
Averaged Concentration [ug/ml]	148.93		209.98	
Total protein in concentrated fraction	77.45		121.79	
total volume of CFPS reaction [uL]	456.00		456.00	
Original concentration [ug/uL]	0.17		0.27	
	Averaged concentration [mg/mL]	0.22		

Genes and plasmid sequences used in this study:

deGFP:

ATGGAGCTTTTCACTGGCGTTGTTCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGT
CCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCG
TGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCA
GCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAA
CTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTT
CAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCG
ACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCC
GACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAG
TCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATC
TAA

deGFP (N terminus 6xhis)

ATGGGCAGCAGCCATCATCATCATCATCACAGCTCTAGAGAGCTTTTCACTGGCGTTGTTCCCATCCTGGTCGAGC
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ACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCG
TGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGT
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CCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGT
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GCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCG
TGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACA
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Methanosarcina mazei PylRS:

ATGGATAAAAAACCACTAAACACTCTGATCTCTGCTACTGGTCTGTGGATGAGTCGTACCGGAACCATTCATAAAA
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TGAGGATCTGAACAAATTCCTGACAAAAGCCAATGAGGACCAAACAAGCGTGAAAGTGAAAGTCGTTAGCGCTCC
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GTTGTACTCGTGAGAACCTGGAAAGCATCATCACCGATTTTCTGAACCACCTGGGCATTGACTTCAAAATTGTGGG
CGACAGCTGTATGGTGTATGGCGACACCCTGGATGTCATGCACGGCGACCTGGAACTGTCTAGTGCCGTTGTTGGA
CCAATTCCGCTGGACCGTGAGTGGGGTATCGACAAACCGTGGATCGGAGCAGGATTCGGTCTGGAACGCCTGCTG
AAAGTGAAACACGACTTCAAAAACATCAAACGTGCCGCCCGTTCTGAATCGTATTATAACGGGATTTCTACCAAC
CTGTAA

Methanosarcina mazei Pyl-tRNA$_{cua}^{pyl}$:

TATGCATGGCGATATCTAATACGACTCACTATAGGAAACCTGATCATGTAGATCGAATGGACTCTAAATCCGTTCA
GCCGGGTTAGATTCCCGGGGTTTCCGCCA

REFRENCES:

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