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Supporting Information

Improving Cell-Free Protein Synthesis through Genome Engineering of *Escherichia coli* Lacking Release Factor 1

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Supplementary Tables

Table S1. Primers used for MAGE, MASC PCR, and DNA sequencing. Underlined bold text indicates mismatch and frameshift insertion. Four bases at 5'-MAGE oligos were phosphorothioated (*).

Primer Name	Primer Sequence (listed 5' to 3')
MAGE	
rna-MAGE	G*C*T*G*ATTTTCTGACCGTACATGGTCTGTGGCCAGGA**ATGTAAATGCTGATGTA**TTGCCTAAATCGGTTGCTGCCCGTGGTGTTGATGAAC
csdA-MAGE	G*C*T*C*TGGCTGATGTCAGGACGGGTAGTCACGCT**TCAGTTA**AATGCGCACTTCCTGCGGCTCTTTCATAAAGCGGCGGGTAATGCGACGA
rnb-MAGE	A*T*T*T*TGTCACCATCGACAGTGCCAGCACAGAAGATATGGAT**TAACTGA**CTTTTCGCTAAGGCGTTGCCGGATGACAAACTTCAGCTGAT
mazF-MAGE	T*T*G*A*TTGCGTTGTACAAGGAACACACAGACACATACCTGTTTTGTTGTT**TCAGTTA**GAAAGGACTCAGGACAACAGCTGGACGATGTCC
endA-MAGE	C*G*G*T*AAAAGTCCACGCTGACGCGCCCGGTACGTTTTATTGC**TAACTGA**AAAATTAACTGGCAGGGCAAAAAAGGCGTTGTTGATCTGCA
MASC PCR	
rna-wt-F	GTACATGGTCTGTGGCCAGGATTGC
rna-mut-F	GGCCAGGA**ATGTAAATGCTGATGTA**
rna-R	TGGCATGACTTCACTTAGTTTAGC
csdA-wt-F	GCCGCAGGAAGTGCGCATTCAGTCC
csdA-mut-F	GCCGCAGGAAGTGCGCATT**TAACTGA**
csdA-R	CAGTGCGCGGTATTGATCCAGATCGC
rnb-wt-F	CCAGCACAGAAGATATGGATGACGCC
rnb-mut-F	CCAGCACAGAAGATATGGAT**TAACTGA**
rnb-R	TCACTTTCAGGCTGCCAGTCACCGG
mazF-wt-F	CTGTTGTCCTGAGTCCTTTCATGTAC
mazF-mut-F	CTGTTGTCCTGAGTCCTTTC**TAACTGA**
mazF-R	GGCTTTAATGAGTTGTAATTCCTCTG
endA-wt-F	CCCGGTACGTTTTATTGCGGATGT
endA-mut-F	CCCGGTACGTTTTATTGC**TAACTGA**
endA-R	GCTGGCGCTGGTAATTTCGGCGTCA
***prfA* deletion confirmation**	
prfA-F	TGATCTGCAAAGCATCATTTCG
prfA-R	TTGCCTCACGTAACCAGTGTTGATA
prfA-R2	CATAACGGCTGTACATACGGAACAG
CAT plasmid construction	
pY71-FW	GGGCGTAAGTCGACCGGCTGCTA
pY71-RV	TTCTCCATATGTATATCTCCTTCTTAAAGTT
CAT-FW	ATATACATATGGAGAAAAAAATCACTGG
CAT-RV	CGGTCGACTTACGCCCCGCCCTG
DNA sequencing	

rna-seq-F	GTTTCTCTGCTTCCCTTCTCTTCT
csdA-seq-F	CTGCTGGACCACCTGAAACGTGGCA
rnb-seq-F	CTGAAAGGCGATCGTTCTTTCTATG
mazF-seq-F	GTAAAGAGCCCGTATTTACGCTTGC
endA-seq-F	ATGTACCGTTATTTGTCTATTGCTGC
mRNA stability	
T7-pro-F	TCGATCCCGCGAAATTAATACGACTCACTATAGG
T7-ter-R	CAAAAAACCCCTCAAGACCCGTTTA
Orthogonal tRNA amplification	
T7tRNA500-F	CCGAAGGTAACTGGCTTCAGCAGAG
T7tRNAopt-R	TGGTCCGGCGGAGGGGATTTGAACCCCTG

Table S2. Strains and plasmids used in this study. Km^R, Sp^R Ap^R, and Cm^R are kanamycin, spectinomycin, ampicillin, and chloramphenicol resistance, respectively. 'Δ' indicates deleted gene, and superscript '-' indicates disabled gene via MAGE.

Strains and plasmids	Genotype/relevant characteristics	Source
Strains		
EcNR2	*MG1655 with λ-prophage::bioA/bioB* and *cmR::mutS*	[1]
BL21 (DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm]* Δ*hsdS λ DE3 = λ sBamHIo* Δ*EcoRI-B int::(lacI::PlacUV5::T7 gene1) i21* Δ*nin5*	New England Biolabs
BL21 Star™ (DE3)	F^- *ompT* $hsdS_B$ ($r_B^-m_B^-$) *gal dcm rne131* (DE3)	Life Technologies
rEc.E13.ΔprfA	Δ*prfA* Ω Sp^R, Ap^R, Cm^R, EcNR2 derivative with 13 TAG termination reassigned to TAA at *coaD, hda, hemA, mreC, murF, lolA, lpxK, yafF, pfpA, sucB, fabH, fliN,* and *atpE*	[2]
MCJ.340	*rEc.E13.ΔprfA rna*$^-$	This study
MCJ.435	*rEc.E13.ΔprfA rnb*$^-$	This study
MCJ.436	*rEc.E13.ΔprfA csdA*$^-$	This study
MCJ.437	*rEc.E13.ΔprfA mazF*$^-$	This study
MCJ.495	*rEc.E13.ΔprfA endA*$^-$	This study
MCJ.438	*rEc.E13.ΔprfA rnb*$^-$ *mazF*$^-$	This study
MCJ.527	*rEc.E13.ΔprfA csdA*$^-$ *rnb*$^-$	This study
MCJ.526	*rEc.E13.ΔprfA csdA*$^-$ *mazF*$^-$	This study
MCJ.560	*rEc.E13.ΔprfA rnb*$^-$ *endA*$^-$	This study
MCJ.559	*rEc.E13.ΔprfA csdA*$^-$ *endA*$^-$	This study
MCJ.561	*rEc.E13.ΔprfA mazF*$^-$ *endA*$^-$	This study
MCJ.485	*rEc.E13.ΔprfA csdA*$^-$ *rnb*$^-$ *mazF*$^-$	This study
MCJ.537	*rEc.E13.ΔprfA csdA*$^-$ *rnb*$^-$ *mazF*$^-$ *endA*$^-$	This study
Plasmids		
pY71-sfGFP	Km^R, P_{T7}::super folder *gfp (sfGFP)*, C-terminal strep-tag	[3]
pY71-sfGFP-T216amb	pY71-sfGFP with amber codon at T216	[3]
pY71-sfGFP-2amb	pY71-sfGFP with amber codon at N212 and T216	[4]
pY71-sfGFP-5amb	pY71-sfGFP with amber codon at D36, K101, E132, D190, and E213	[4]
pY71-CAT	Km^R, P_{T7}::CAT (chloramphenicol acetyl transferase)	This study
pY71-CAT-D112amb	pY71-CAT with amber codon at D122	This study
pY71-pAcFRS	P_{T7}::pAcFRS, C-terminal 6x histidine tag	[4]
pY71-mRFP1-Spinach	P_{T7}::mRFP1-Spinach aptamer	[5]
pDULE-o-tRNA	P_{lpp}::o-tRNA, Tet^R	[3]
pY71-T7-tz-o-tRNA^{opt}	P_{T7}:: hammer-head ribozyme, o-tRNA^{opt}	[4]

Table S3. Doubling time of MAGE strains. Cells were grown in 2xYTPG medium at 34℃ in a 96-well plate.

Strains	Doubling time (min)	Percentage (%)
rEc.E13.ΔprfA	34.5 ± 0.4	100
MCJ.340	38.1 ± 0.4	110
MCJ.435	43.8 ± 0.8	127
MCJ.436	41.7 ± 0.4	121
MCJ.437	32.4 ± 0.0	94
MCJ.438	52.5 ± 0.4	152
MCJ.527	42 ± 2	121
MCJ.526	40 ± 1	116
MCJ.485	38 ± 1	111
MCJ.495	34 ± 1	97
MCJ.560	39 ± 1	114
MCJ.559	36.6 ± 0.6	106
MCJ.561	33.0 ± 0.8	96
MCJ.537	39.5 ± 0.9	114

Table S4. Monoisotopic masses calculated from mass spectrometric data. 'Experiment' indicates experimentally obtained protein mass, and 'Theoretical' indicates theoretically calculated protein mass.

sfGFP species	Mass (Da, Experiment)	Mass (Da, Theoretical)	Error (ppm)	Shift from WT-sfGFP (Da, Experiment)	Shift from WT-sfGFP (Da, Theoretical)
WT-sfGFP	26847.50	26847.45	2.6	-	-
sfGFP-1pAcF (T216)	26935.54	26935.48	2.2	88.04	88.03
sfGFP-2pAcF (N212, T216)	27010.56	27010.52	1.5	163.06	163.07
sfGFP-5pAcF (D36, K101, E132, D190, E213)	27176.66	27176.61	1.8	329.16	329.16

Supplementary Figures

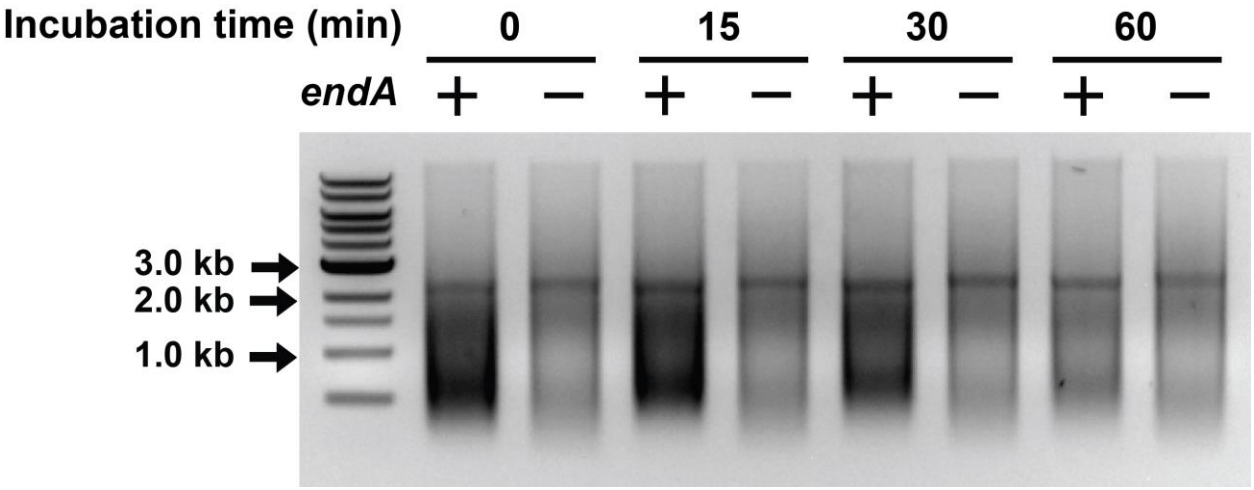

Figure S1. DNA gel to assess degradation of plasmid DNA in crude extracts with or without endonuclease I. pY71-sfGFP plasmid DNA was incubated with cell extracts from *rEc.E13.ΔprfA* (*endA*$^{+}$) and MCJ.495 (*endA*$^{-}$) strains for 0, 15, 30 and 60 min at 30℃. Samples were RNase A treated and purified using phenol:chloroform:isoamyl alcohol solution followed by DNA purification kit. The purified plasmid DNA was linearized by BamHI digestion prior to loading on agarose gel (0.7%). Expected band size was 2.5 kb.

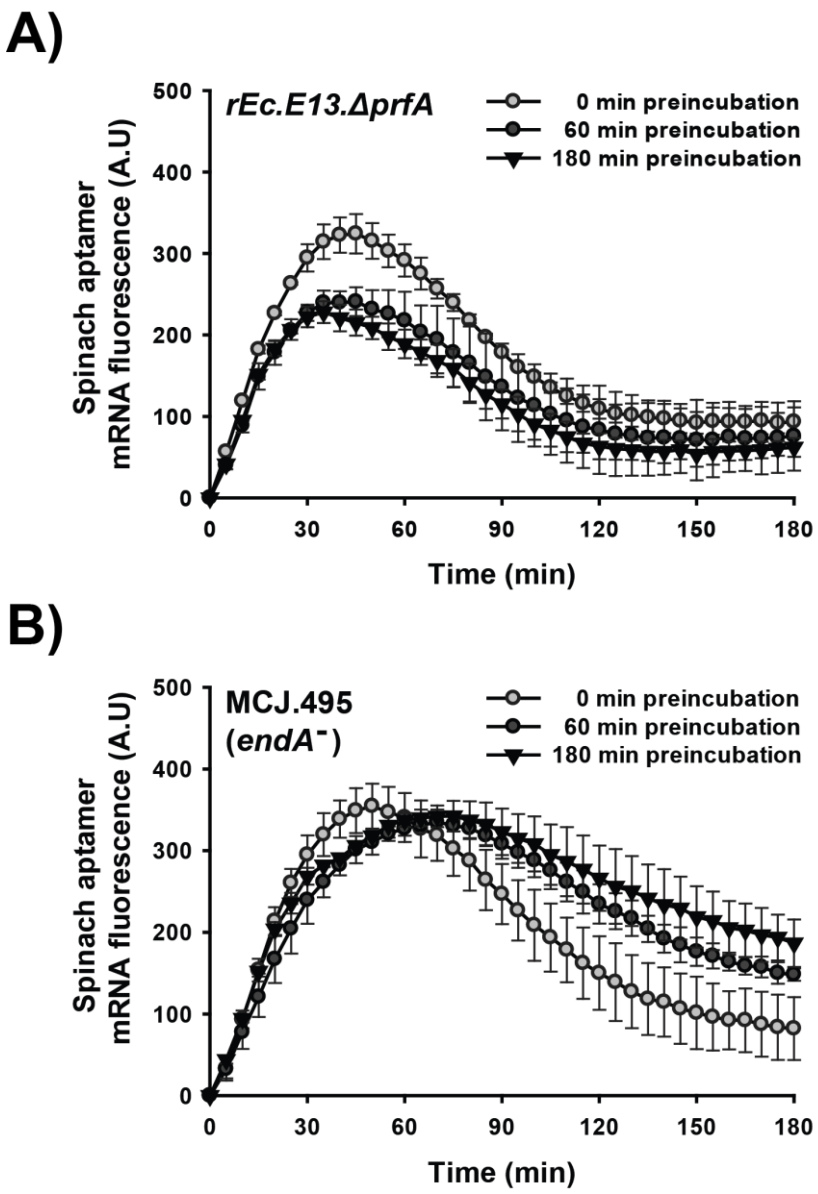

Figure S2. Time course Spinach aptamer mRNA synthesis using A) endonuclease I present (*rEc.E13.ΔprfA*) and B) deficient (MCJ.495) extract. Plasmid DNA containing Spinach aptamer gene was preincubated for 0, 60, and 180 min with cell extract and DFHBI. CFPS reactions were performed upon adding CFPS reagents and monitored for 180 min. Maximum mRNA fluorescence was taken for the comparison of mRNA stability in Figure 2B of the main text. At least three independent reactions for each sample were performed at 30℃. One standard deviation is shown.

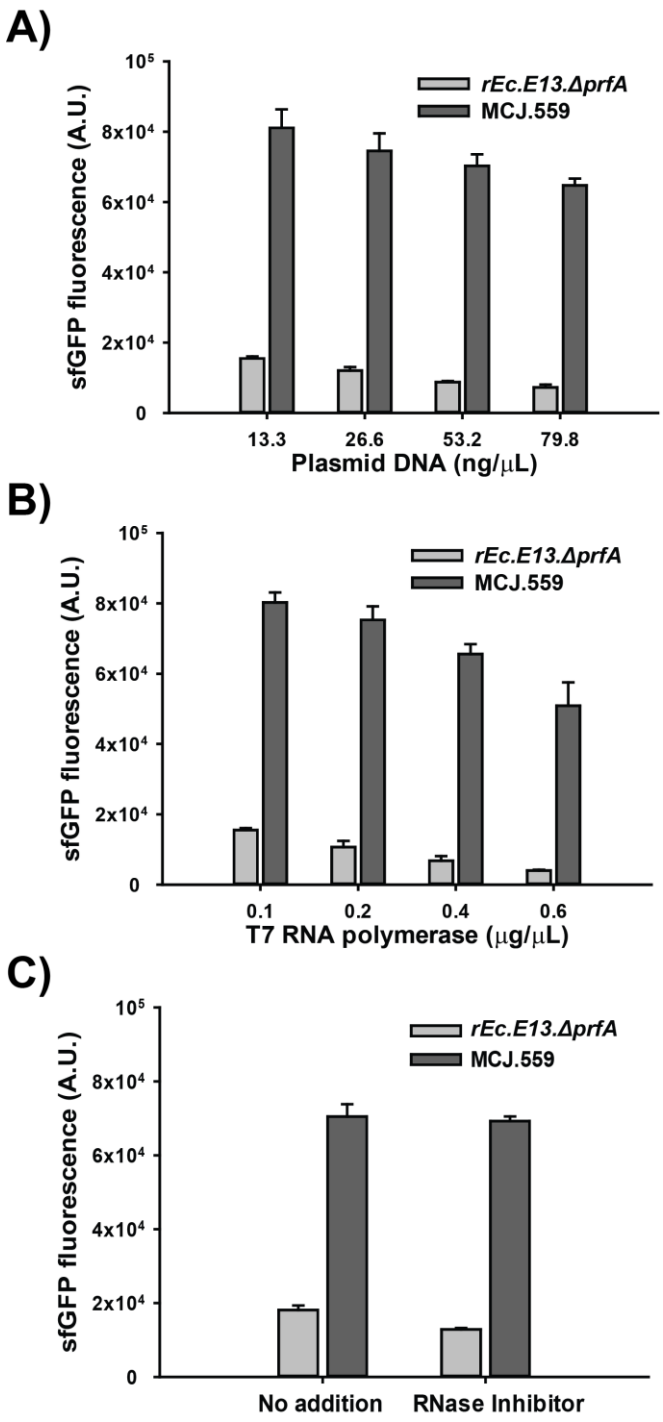

Figure S3. Optimization of plasmid DNA and T7 RNA polymerase and the effect of RNase inhibitor in CFPS reactions. CFPS yields observed by increasing the concentration of **A)** plasmid DNA and **B)** T7 RNA polymerase using the extracts from *rEc.E13.ΔprfA* and MCJ.559. **C)** CFPS yields observed in the presence or absence of RNase inhibitor. At least three independent reactions for each sample were performed for 20 h at 30℃. One standard deviation is shown.

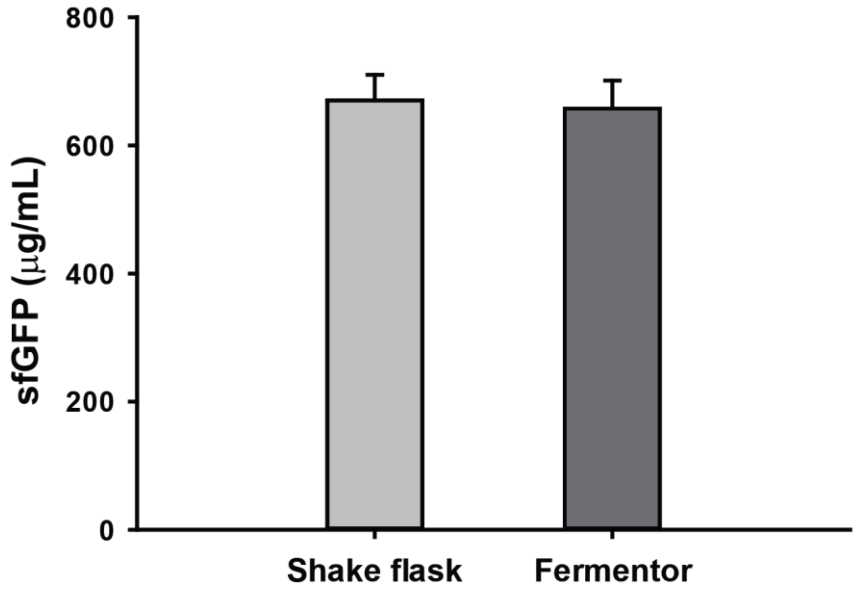

Figure S4. Cell extract performance comparison between shake flask- and fermentor-grown MCJ.559. sfGFP synthesis was quantified by measuring the fluorescence in CFPS reactions. Three independent reactions for each sample were performed for 20 h at 30℃. One standard deviation is shown.

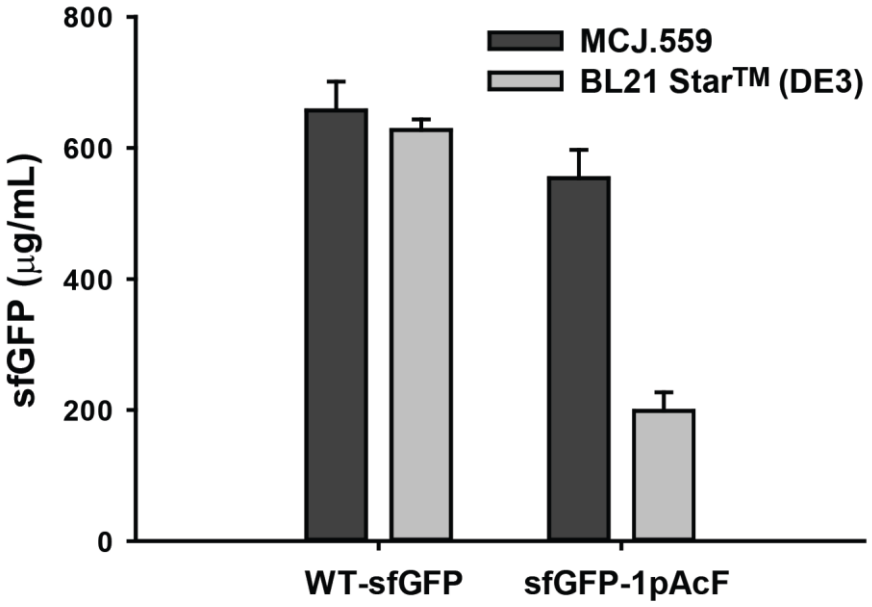

Figure S5. Cell extract performance comparison between MCJ.559 and BL21 Star™ (DE3) extract. Active sfGFP synthesis was quantified by measuring fluorescence in CFPS reactions. Single pAcF was incorporated to the amber position corresponding to T216 of sfGFP. Three independent reactions for each sample were performed for 20 h at 30℃. One standard deviation is shown.

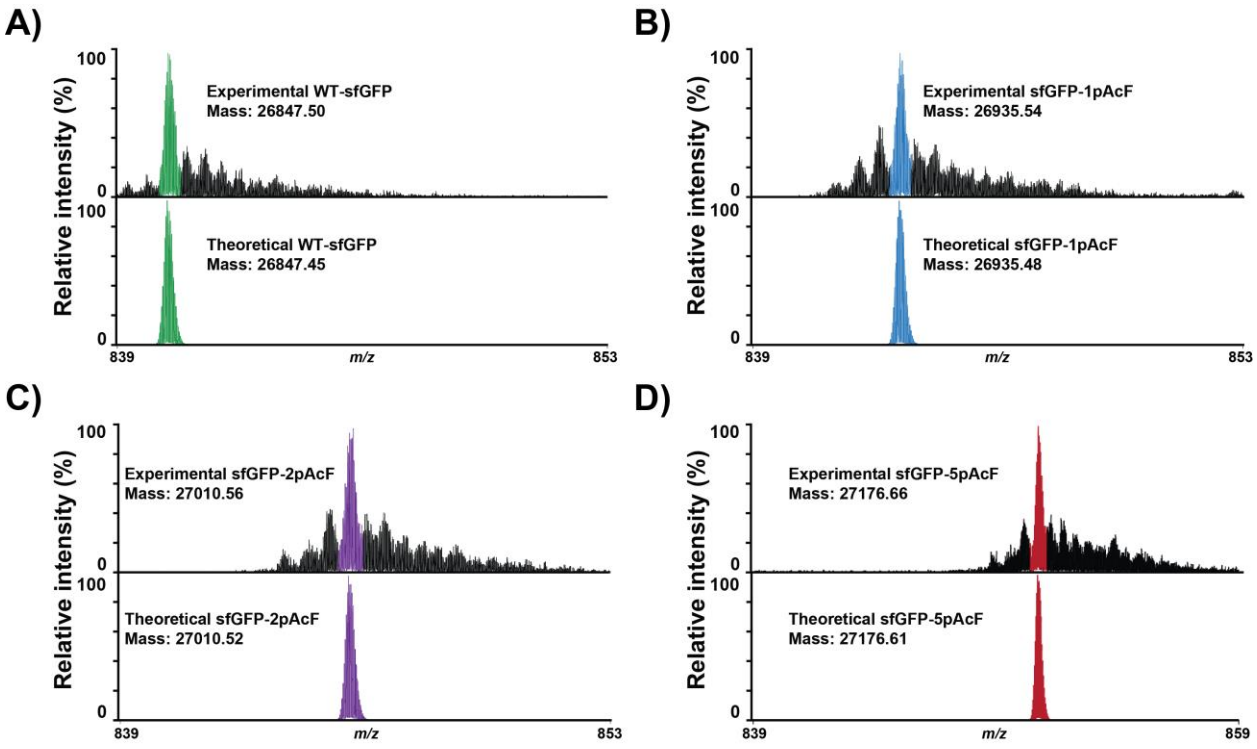

Figure S6. Mass spectrometry analysis of pAcF incorporation for 1, 2, and 5 amber sites. Experimental and theoretical mass peaks of **A)** wild-type sfGFP, **B)** sfGFP containing single pAcF corresponding to the position of T216, **C)** sfGFP containing two pAcF corresponding to the positions of N212 and T216, and **D)** sfGFP containing five pAcF corresponding to the positions of D36, K101, E132, D190, and E213.

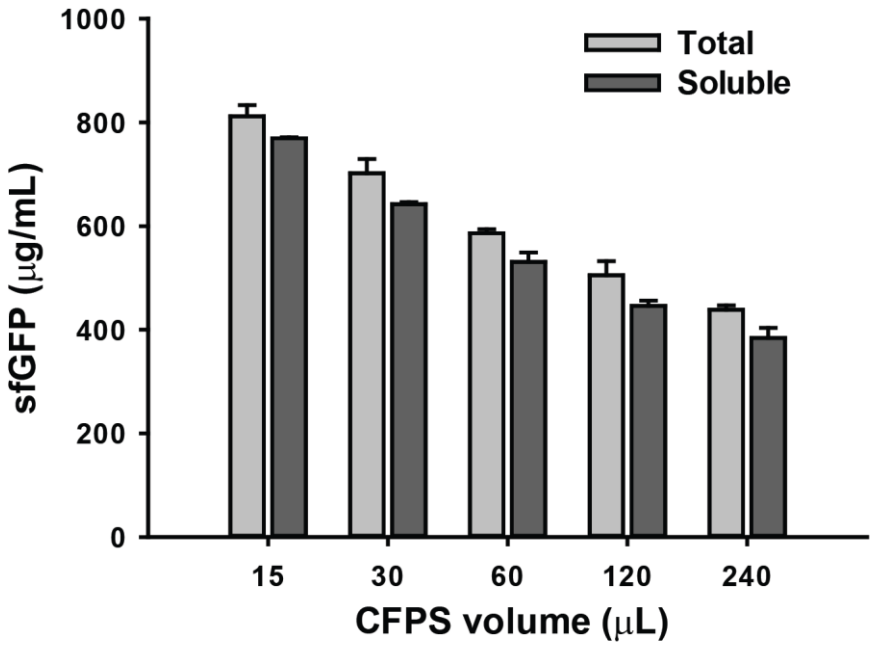

Figure S7. Radioactive ^{14}C-Leu incorporation of scale up CFPS reactions using MCJ.559 extract indicates decreasing protein expression yields with increasing reaction volume. Wild-type sfGFP was synthesized in different reaction volumes using a microcentrifuge tube. The total and soluble protein production was assessed by counting radioactive ^{14}C-Leu incorporation. At least three independent reactions for each sample were performed for 20 h at 30℃. One standard deviation is shown.

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