Supporting Information

Cell-free protein synthesis from a release factor 1 deficient *Escherichia coli* activates efficient and multiple site-specific non-standard amino acid incorporation

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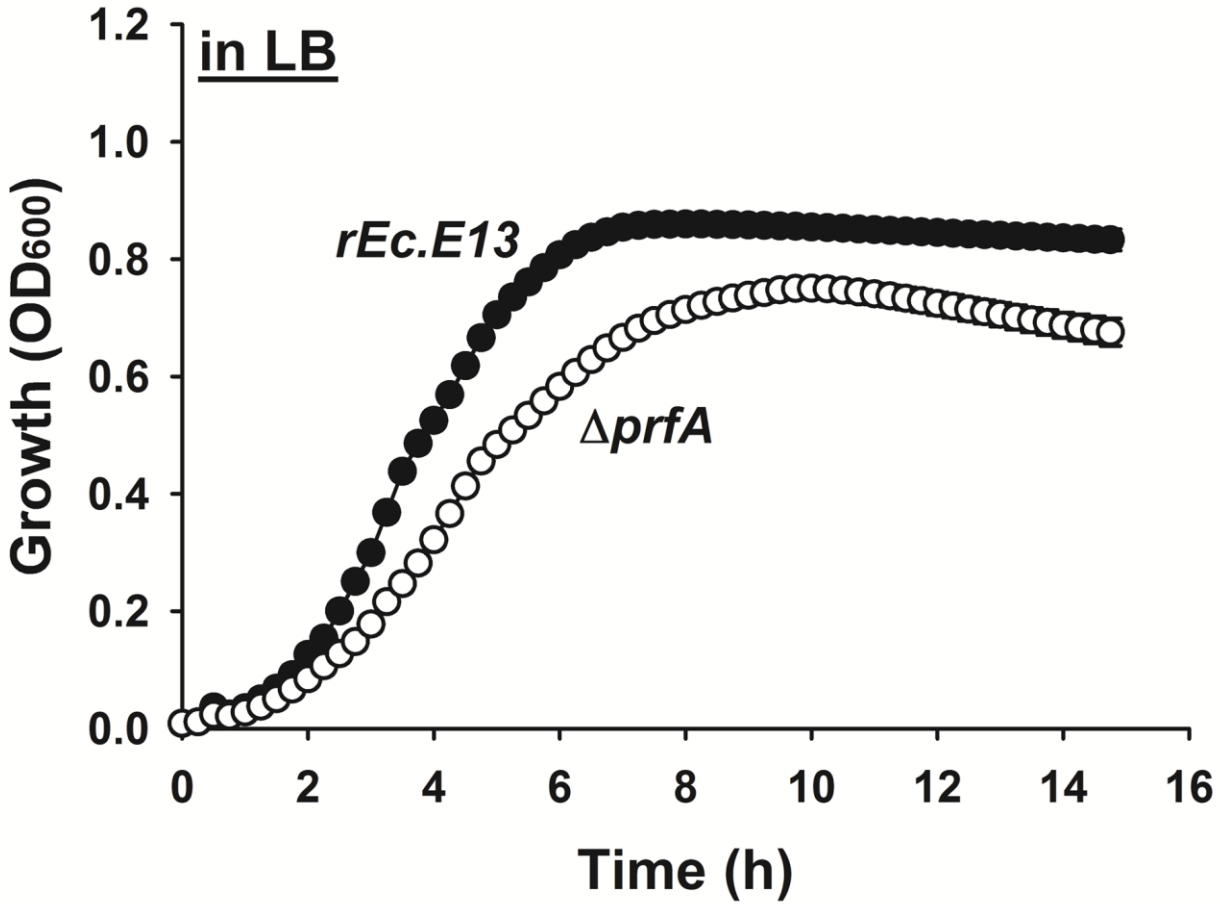
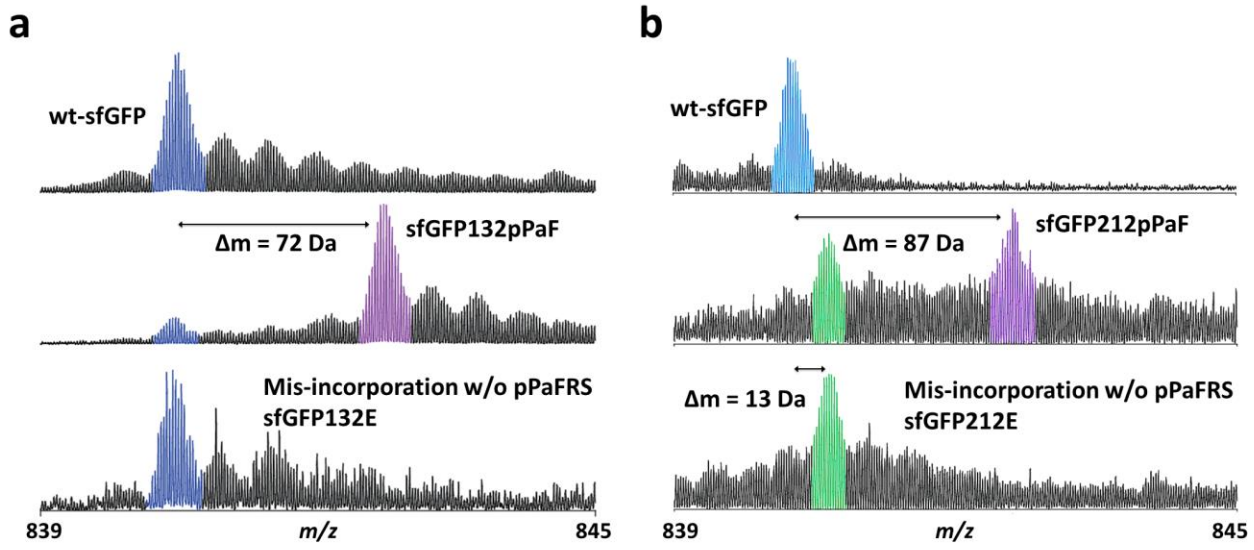
Supporting Table S1. Primers used for DNA sequencing, plasmid construction, and PCR amplification. Primers for amber codon insertion were phosphorylated at 5' end. Bold text in the primers for amber codon insertion indicates amber codon (TAG). Underlined bold text indicates BglII restriction site in T7-PCRamp-f, SalI restriction site in T7-PCRamp-r and pAcF-SalI-r, and NdeI restriction site in pAcF-NdeI-f. Underlined italic text indicates 6x His-tag sequence in pAcF-SalI-r.

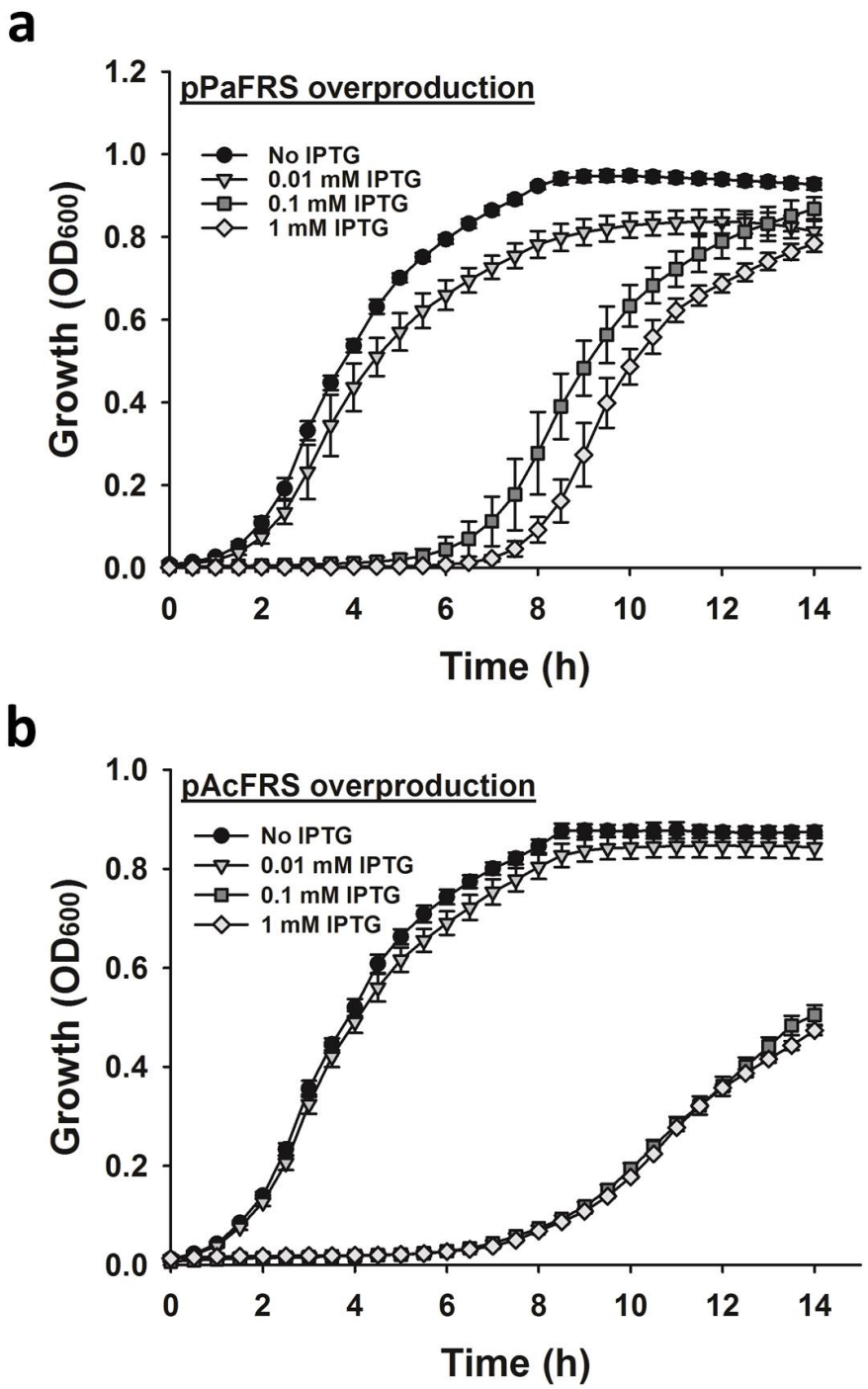
Primer Name	Primer Sequence (listed 5' to 3')
DNA sequencing	
pY71-f	GAGCCTATGGAAACGAATTCAGATC
pY71-r	TTTCTAATCAGAATTGGCTTTCAGC
tRNA-seq-f	TACAGCGTGAGCATTGAGAAAGCGC
tRNA-seq-r	GCCTCGGTGAGTTTTCTCCTTCATT
Amber codon insertion	
TAG-E132-f	/5'Phos/**TAG**GATGGCAATATCCTGGGCCATAAACTG
TAG-E132-r	/5'Phos/TTTAAAATCCGTGCCTTTCAGTTCAATGCG
TAG-N212-f	/5'Phos/**TAG**GAAAAAGGCACGCGGGACCACATGG
TAG-N212-f2	/5'Phos/**TAG**GAAAAAGGC**TAG**CGGGACCACATGG
TAG-N212-r	/5'Phos/CGGATCTTTAGACAGAACGGTCTGC
Cloning	
T7-PCRamp-f	GCTTTT**AGATCT**TAATACGACTCAC
T7-PCRamp-r	GCTTTTG**GTCGAC**GGATGTAAGCTTCC
pAcF-NdeI-f	GAGATATA**CATATG**GACGAATTTGAAATGATAAAGAGA
pAcF-SalI-r	AGCAGCCG**GTCGAC**TTATTA*GTGATGGTGATGGTGATG*TAATCTC TTTCTAATTGGCTC
Linear o-tRNA	
T7tRNA500-f	CCGAAGGTAACTGGCTTCAGCAGAG
T7tRNAopt-r	TGGTCCGGCGGAGGGGATTTGAACCCCTG
T7tRNA-r	TGGTCCGGCGGGCCGGATTTGAACCAGCG



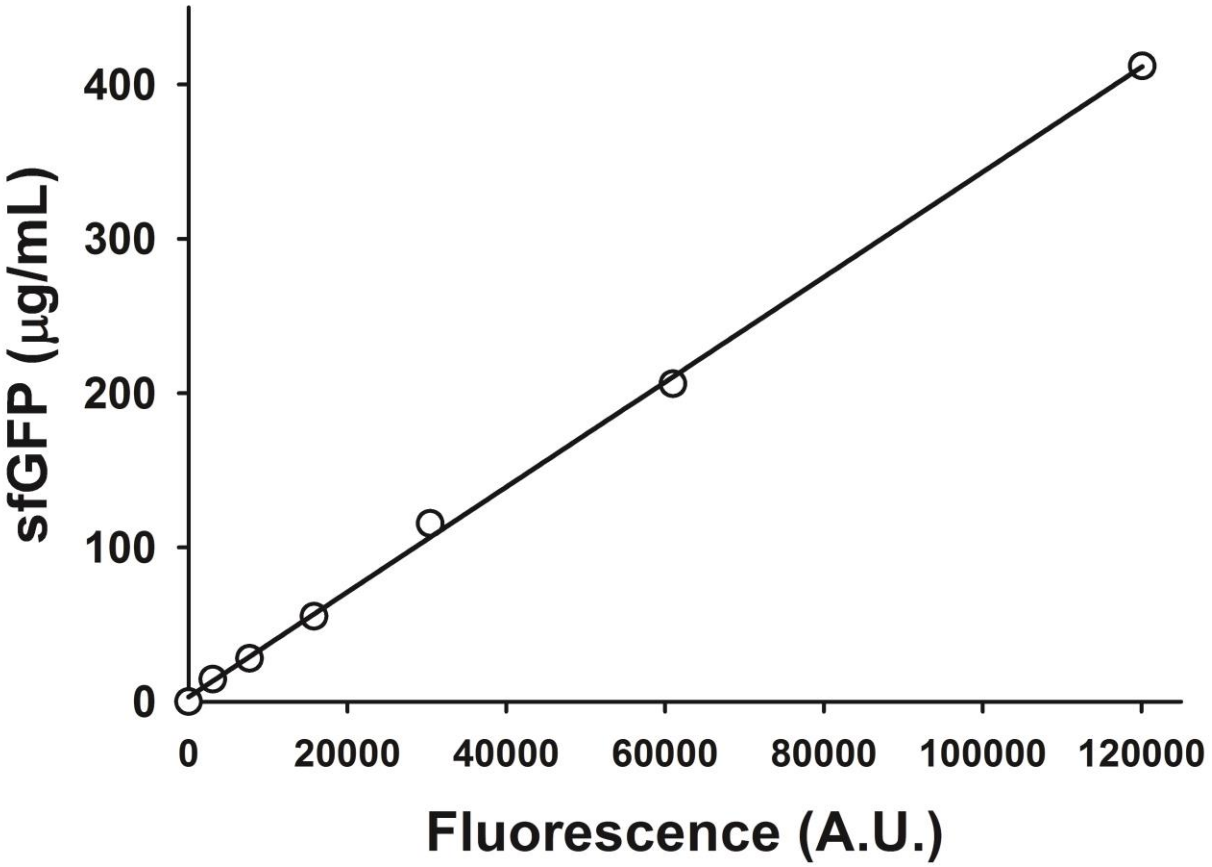
Supporting Figure S1. Growth of *rEc.E13* and Δ*prfA*. Cells were grown in LB medium at 34℃ in 96 well plates. Each data point is the average of ten replicate wells from two independent cultures.



Supporting Figure S2. Mass spectrometry analysis of pPaF incorporation at single amber site. pPaF incorporation was examined to the amber site corresponding to the position of E132 (**a**) and N212 (**b**). mis-incorporation was examined without adding pPaFRS in CFPS reaction.



Supporting Figure S3. Cell growth with overproducing pPaFRS or pAcFRS shows the possible toxic effect of orthogonal translation components. BL21(DE3) cells harboring pY71-pPaFRS (**a**) or pY71-pAcFRS (**b**) were grown in LB medium with different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 ℃ in 96 well plates. Each data point is the average of six replicate wells from three independent cultures.



Supporting Figure S4. Standard curve for determining sfGFP concentrations. Fluorescence signal was measured upon different concentrations of soluble sfGFP quantified by determining radioactive ^{14}C-Leu incorporation. A.U. is arbitrary unit.