Supplementary data

A Genetically Encoded Fluorescent tRNA Is Active in Live-Cell Protein Synthesis

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Running Title: Spinach tRNA

Supplementary Table S1. Gene sequences for Spinach tRNAs.

Spinach	**5′-GACGCGACTGAATGAAATGGTGAAGGACGGGTCCAGGT GTGGCTGCTTCGGCAGTGCAGCTTGTTGAGTAGAGTGTG AGCTCCGTAACTAGTCGCGTC-3′**
V-Spinach *Ec* tRNA$^{Tyr2/GUA}$	**5′-GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTGTAA ATCTGCCGTCACGACGCGACTGAATGAAATGGTGAAGGA CGGGTCCAGGTGTGGCTGCTTCGGCAGTGCAGCTTGTTG AGTAGAGTGTGAGCTCCGTAACTAGTCGCGTCAGACTTC GAAGGTTCGAATCCTTCCCCCACCACCA-3′**
V-Spinach *Ec* tRNA$^{Tyr2/CUA}$	**5′-GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAA ATCTGCCGTCACGACGCGACTGAATGAAATGGTGAAGGA CGGGTCCAGGTGTGGCTGCTTCGGCAGTGCAGCTTGTTG AGTAGAGTGTGAGCTCCGTAACTAGTCGCGTCAGACTTC GAAGGTTCGAATCCTTCCCCCACCACCA-3′**
V-Spinach *Ec* tRNA$^{Tyr2/CUA}$ No CCA-tail	**5′-GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAA ATCTGCCGTCACGACGCGACTGAATGAAATGGTGAAGGA CGGGTCCAGGTGTGGCTGCTTCGGCAGTGCAGCTTGTTG AGTAGAGTGTGAGCTCCGTAACTAGTCGCGTCAGACTTC GAAGGTTCGAATCCTTCCCCCACCA-3′**
V-Spinach *Ec* tRNA$^{Tyr2/CUA}$ (U71-U72)	**5′-GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAA ATCTGCCGTCACGACGCGACTGAATGAAATGGTGAAGGA CGGGTCCAGGTGTGGCTGCTTCGGCAGTGCAGCTTGTTG AGTAGAGTGTGAGCTCCGTAACTAGTCGCGTCAGACTTC GAAGGTTCGAATCCTTCCCCCATTACCA-3′**
T-Spinach *Ec* tRNA$^{Tyr2/CUA}$	**5′-GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAA ATCTGCCGTCACAGACTTCGAAGGTTCGGACGCGACTGA ATGAAATGGTGAAGGACGGGTCCAGGTGTGGCTGCTTCG GCAGTGCAGCTTGTTGAGTAGAGTGTGAGCTCCGTAACT AGTCGCGTCAATCCTTCCCCCACCACCA-3′**
D-Spinach *Ec* tRNA$^{Tyr2/CUA}$	**5′-GGTGGGGTTCCCGAGCGACGCGACTGAATGAAATGGTGA AGGACGGGTCCAGGTGTGGCTGCTTCGGCAGTGCAGCTT GTTGAGTAGAGTGTGAGCTCCGTAACTAGTCGCGTCGGCC AAAGGGAGCAGACTCTAAATCTGCCGTCACAGACTTCGAA GGTTCGAATCCTTCCCCCACCACCA-3′**

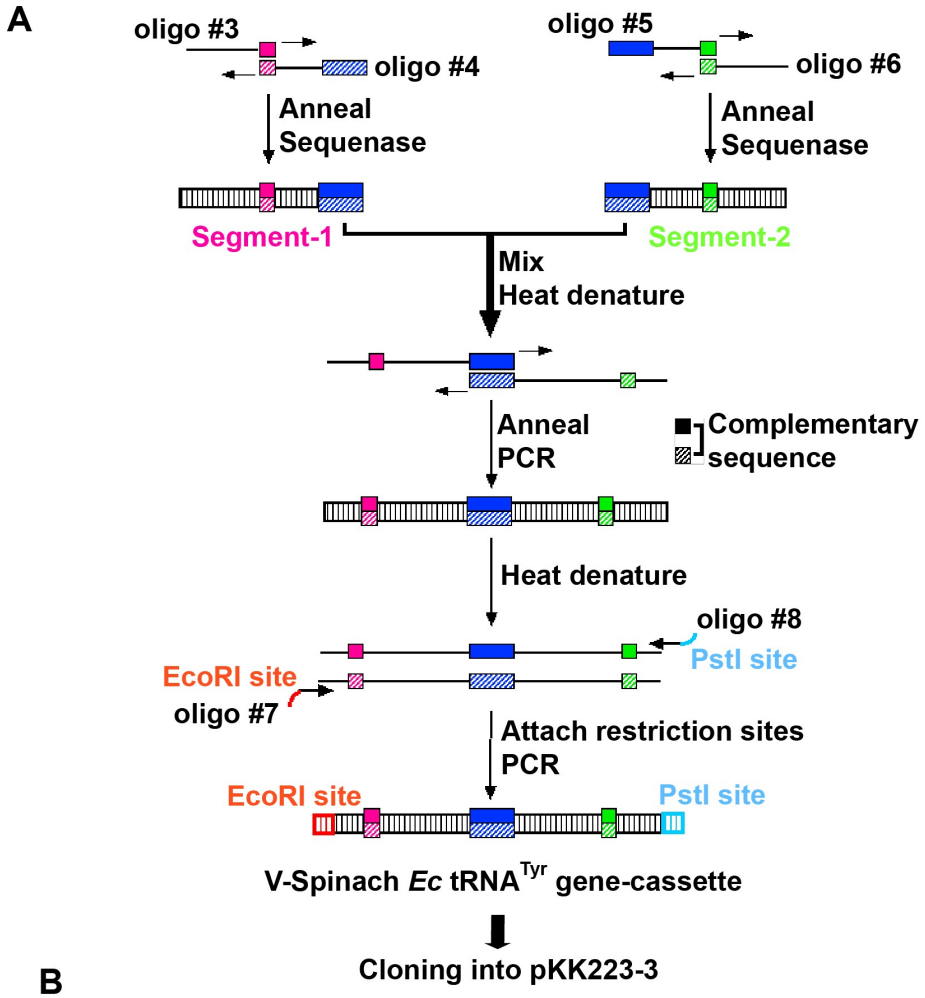
Note that anticodons are shown in red.

Supplementary Table S2. Sequences of oligonucleotides used for construction of Spinach tRNAs.

#1	**5′-TTTTCGCGTCGAATTCGACGCGACTGAATGAAATGGTGA AGGACGGGTCCAGGTGTGGCTGCTTCGGCAGTGC-3′**
#2	**5′-ATTTCGCGTCCTGCAGGACGCGACTAGTTACGGAGCTCA CACTCTACTCAACAAGCTGCACTGCCGAAGCAGCC-3′**
#3	**5′-GACTGACTGACTGGTGGGGTTCCCGAGCGGCCAAAGGG AGCAGACTGTAAATCTGCCGTCACGACG-3′**
#4	**5′-CTGCACTGCCGAAGCAGCCACACCTGGACCCGTCCTTCA CCATTTCATTCAGTCGCGTCGTGACGGC-3′**
#5	**5′-GAAGGACGGGTCCAGGTGTGGCTGCTTCGGCAGTGCAG CTTGTTGAGTAGAGTGTGAGCTCCGTAACTAG-3′**
#6	**5′-CAGTCAGTCAGTTGGTGGTGGGGGAAGGATTCGAACCTT CGAAGTCTGACGCGACTAGTTACGGAGCTCA-3′**
#7	**5′-TTTCCCCACCGAATTCGGTGGGGTTCCCGAGCGGC-3′**
#8	**5′-ACCACCACTGCAGTGGTGGTGGGGGAAGGATTCGAACCTTC-3′**
#9	**5′-GACTGACTGACTGGTGGGGTTCCCGAGCGGCCAAAGGG AGCAGACTCTAAATCTGCCGTCACGACG-3′**
#10	**5′-AAACCCACCACTGCAGTGGTGGGGGAAGGATTCGAACC-3′**
#11	**5′-CAGTCAGTCAGTTGGTAATGGGGGAAGGATTCGAACCTT CGAAGTCTGACGCGACTAGTTACGGAGCTCA-3′**
#12	**5′-AAAATTACCACTGCAGTGGTAATGGGGGAAGGATTCGAAC-3′**
#13	**5′-GACTGACTGACTGGTGGGGTTCCCGAGCGGCCAAAGGGA GCAGACTCTAAATCTGCCGTCACAGACTTC-3′**
#14	**5′-GCCACACCTGGACCCGTCCTTCACCATTTCATTCAGTCGC GTCCGAACCTTCGAAGTCTGTGACGGCAG-3′**
#15	**5′-GACTGAATGAAATGGTGAAGGACGGGTCCAGGTGTGGC TGCTTCGGCAGTGCAGCTTGTTGAGTAGAGTGTG-3′**
#16	**5′-CAGTCAGTCAGTTGGTGGTGGGGGAAGGATTGACGCGA CTAGTTACGGAGCTCACACTCTACTCAACAAGCTG-3′**
#17	**5′-TTTACCACCACTGCAGTGGTGGTGGGGGAAGGATTGAC-3′**
#18	**5′-GACTGACTGACTGGTGGGGTTCCCGAGCGACGCGACTG AATGAAATGGTGAAGGACGGGTCCAGGTGTGGC-3′**
#19	**5′-CGCGACTAGTTACGGAGCTCACACTCTACTCAACAAGC TGCACTGCCGAAGCAGCCACACCTGGACCCG-3′**
#20	**5′-GCAGCTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTC GCGTCGGCCAAAGGGAGCAGACTCTAAATCTGCC-3′**
#21	**5′-CAGTCAGTCAGTTGGTGGTGGGGGAAGGATTCGAACCTT CGAAGTCTGTGACGGCAGATTTAGAGTCTGCTC-3′**
#22	**5′-CCCCACCGAATTCGGTGGGGTTCCCGAGCGACGC-3′**

Supplementary Figure S1.

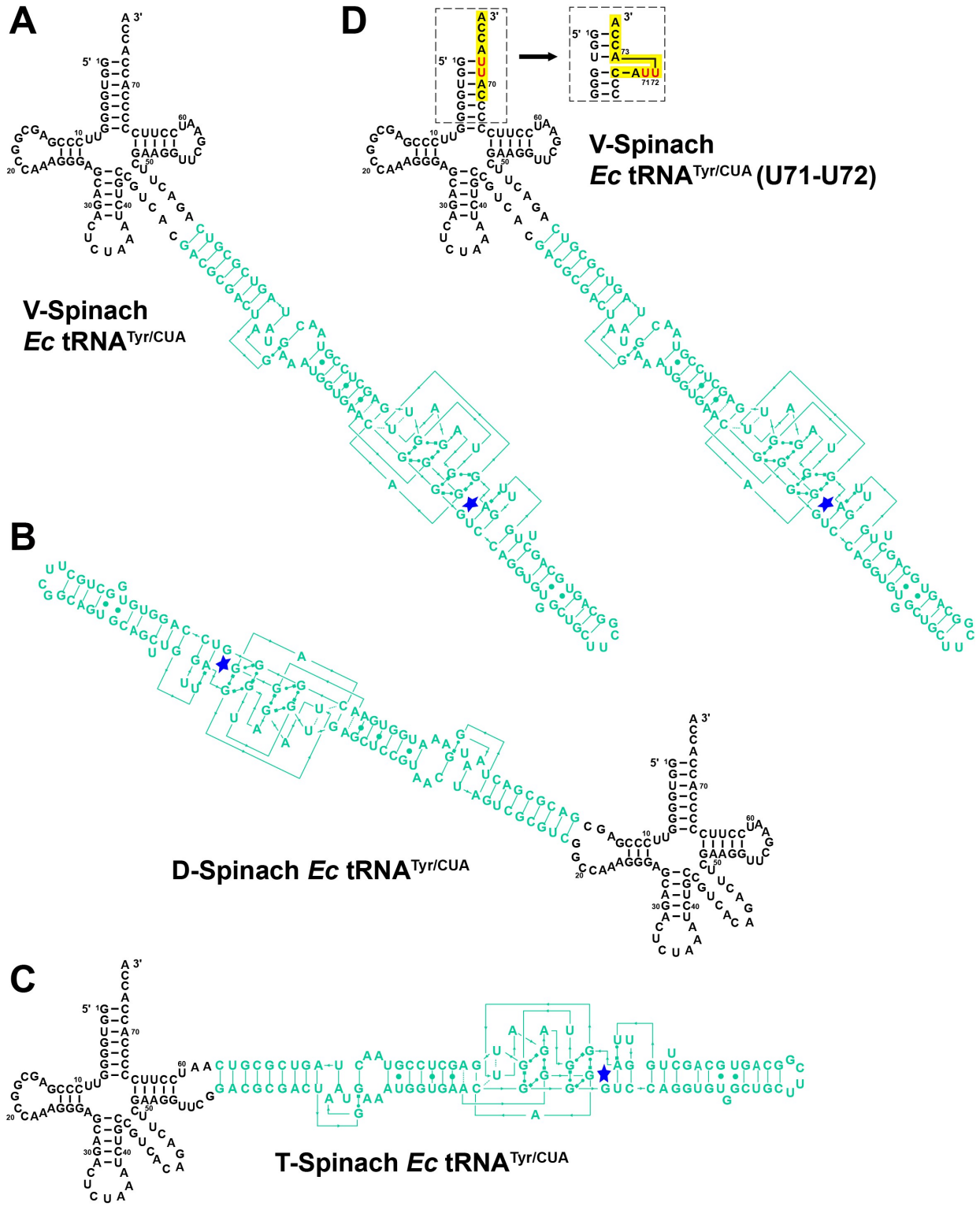
A



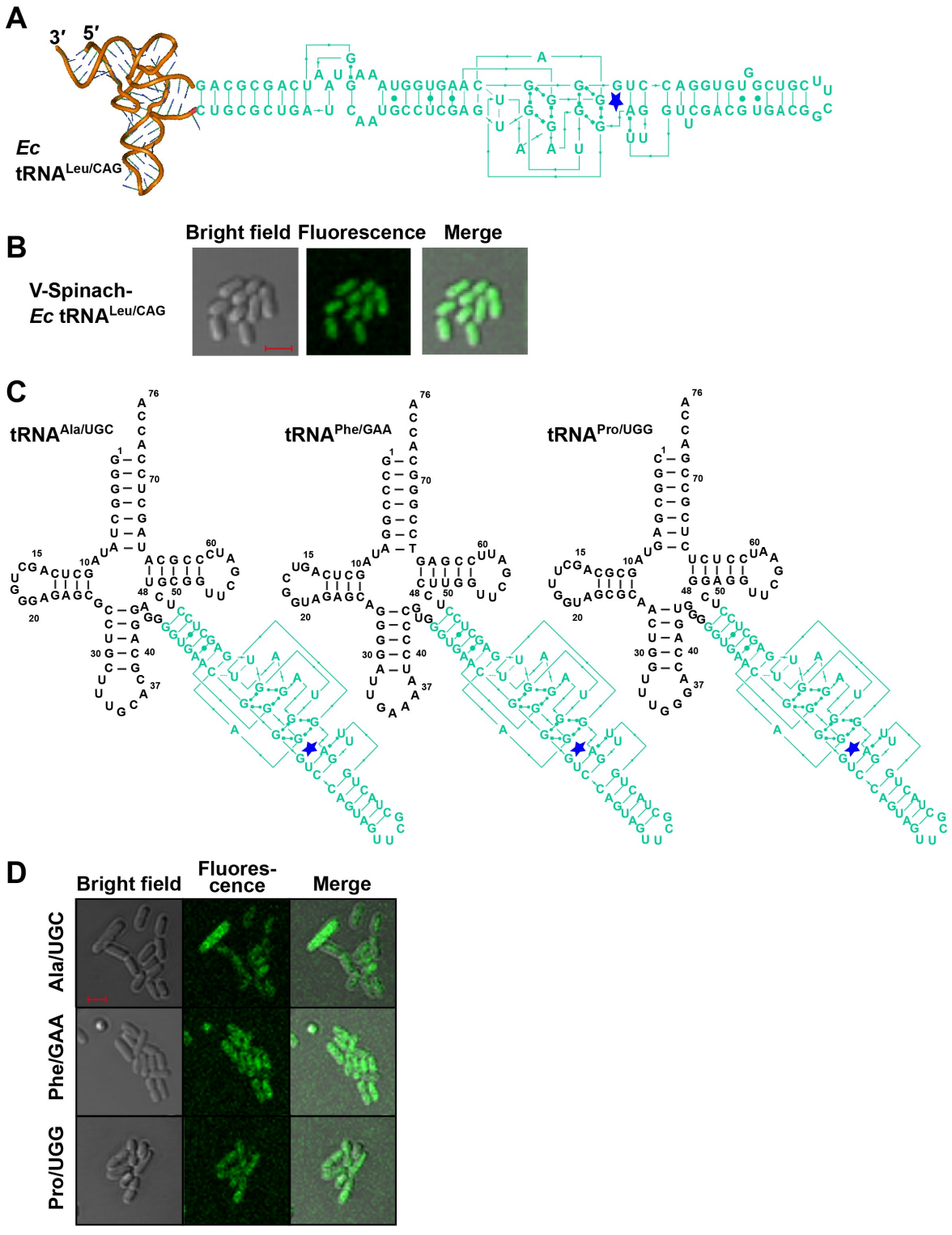
B

RNA		Segment 1	Segment 2	PCR
Spinach	Forward	#1		
	Reverse	#2		
V-Spinach *Ec* tRNA$^{Tyr/GUA}$	Forward	#3	#5	#7
	Reverse	#4	#6	#8
V-Spinach *Ec* tRNA$^{Tyr/CUA}$	Forward	#9	#5	#7
	Reverse	#4	#6	#8
V-Spinach *Ec* tRNA$^{Tyr/CUA}$ No CCA-tail	Forward	#9	#5	#7
	Reverse	#4	#6	#10
V-Spinach *Ec* tRNA$^{Tyr/CUA}$ U71-U72	Forward	#9	#5	#7
	Reverse	#4	#11	#12
T-Spinach *Ec* tRNA$^{Tyr/CUA}$	Forward	#13	#15	#7
	Reverse	#14	#16	#17
D-Spinach *Ec* tRNA$^{Tyr/CUA}$	Forward	#18	#20	#22
	Reverse	#19	#21	#8

Supplementary Figure S2.



Supplementary Figure S3.



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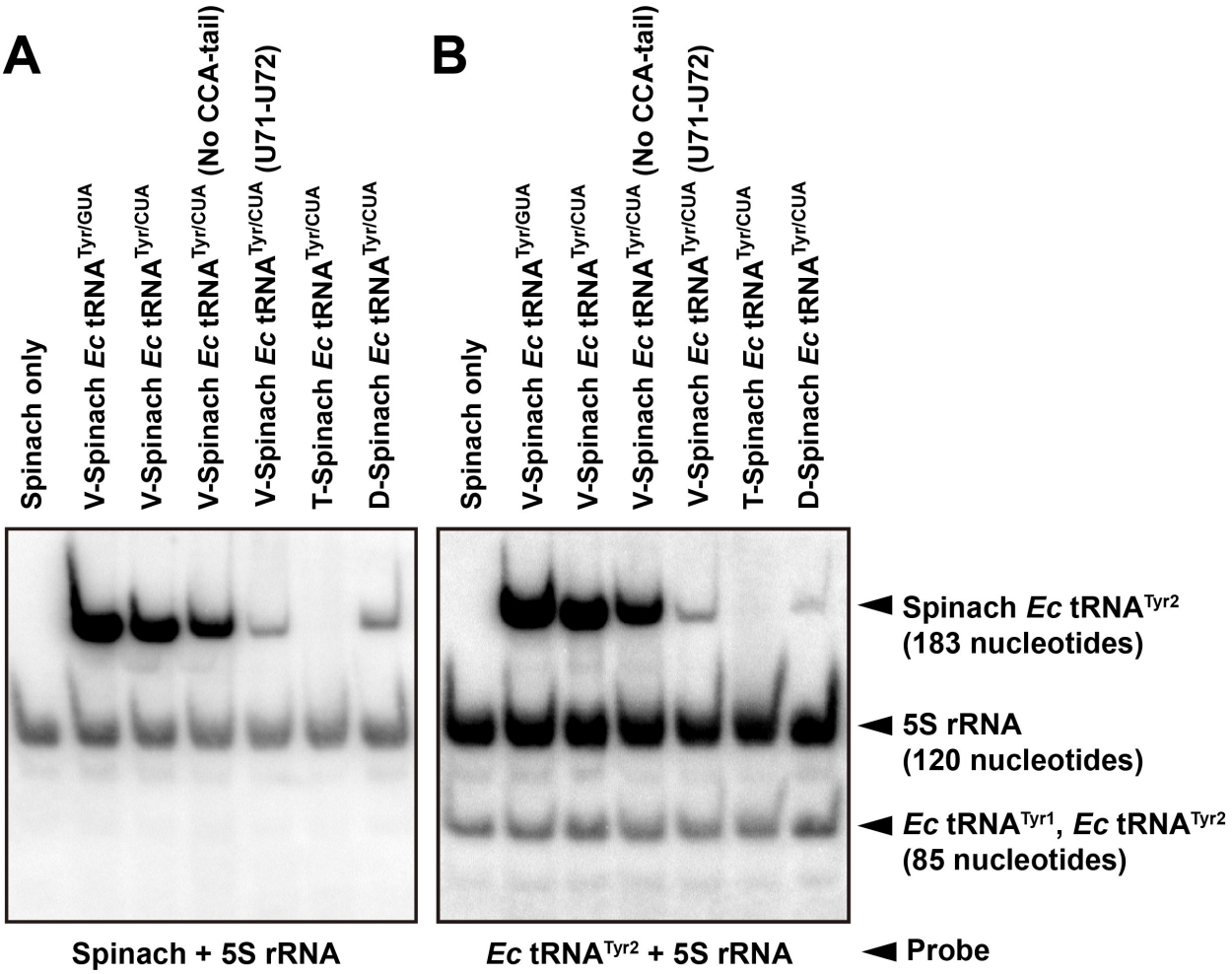

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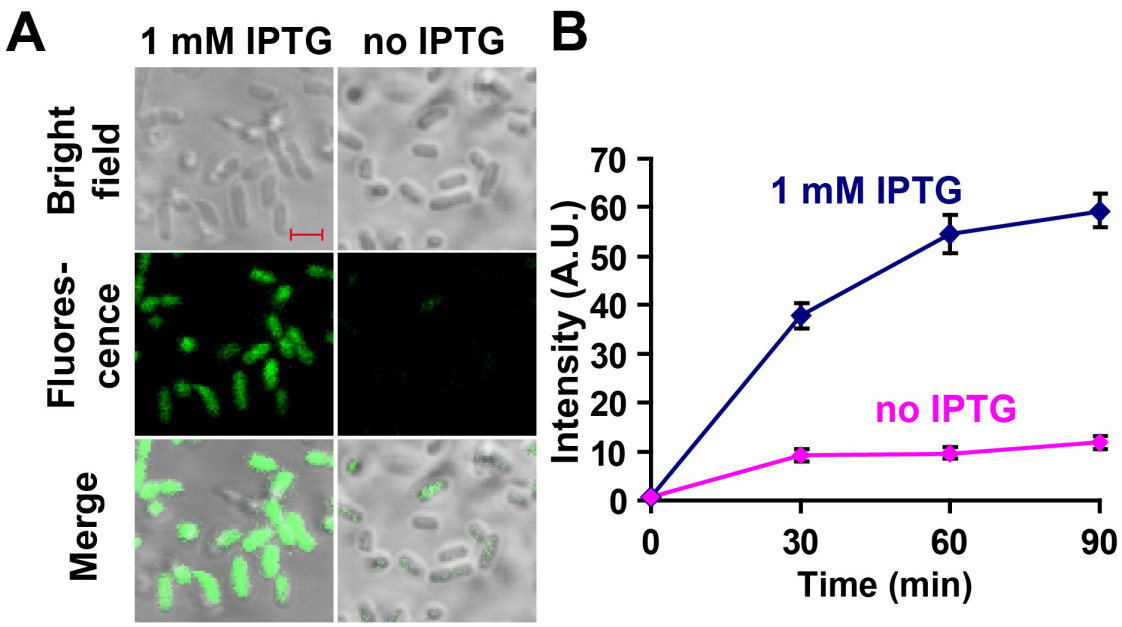
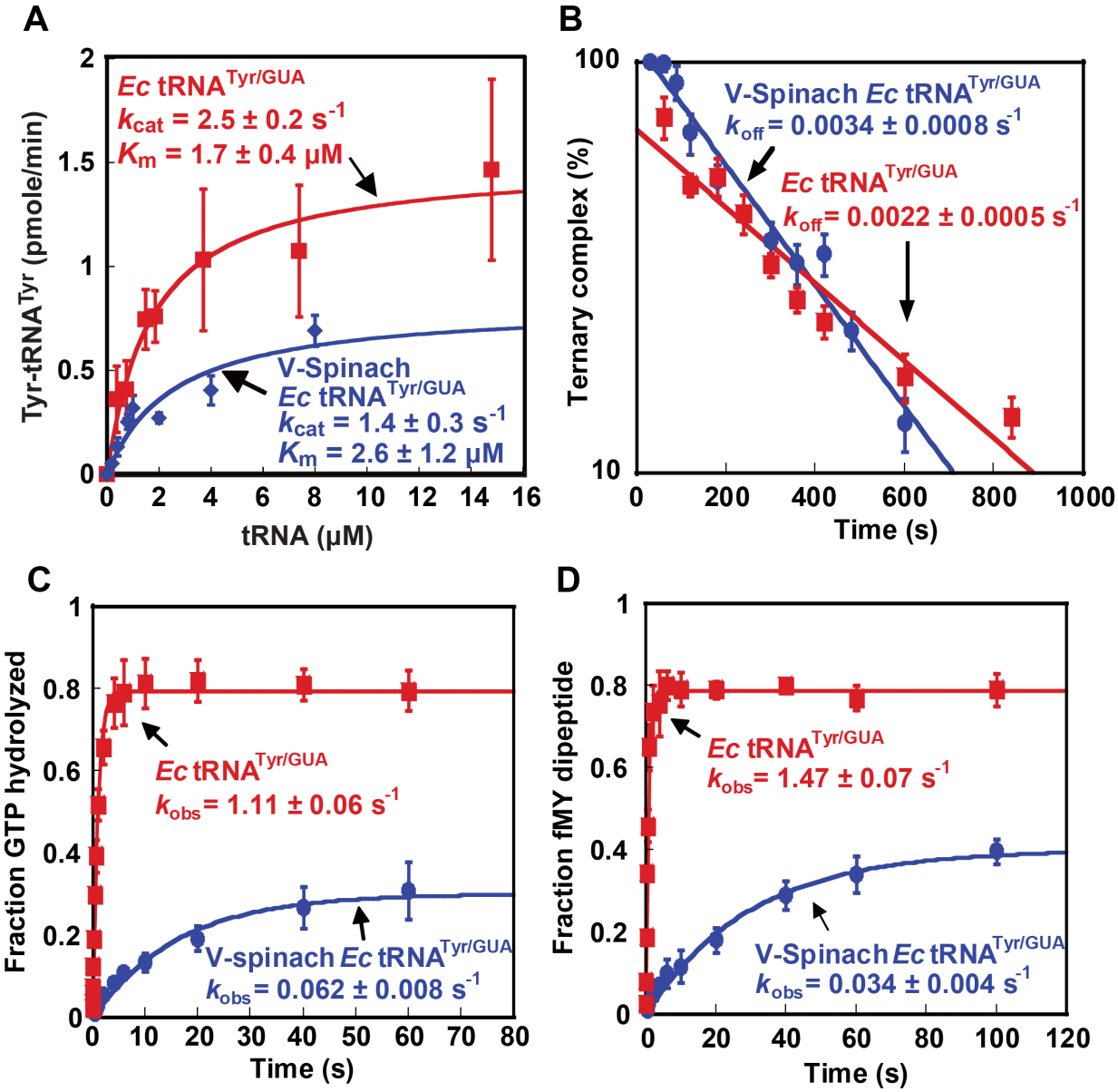


Supplementary Figure S6.



Supporting Information Figure Legends

Supplementary Figure S1. Scheme for synthesizing V-Spinach tRNA^{Tyr}. (**A**) A scheme is presented to describe the construction of V-Spinach tRNA^{Tyr}. Oligo #3 and oligo #4 were annealed and the hybrid was extended by Sequenase to synthesize segment 1, while oligo #5 and oligo #6 were annealed and the hybrid was extended to synthesize segment 2. The two segments were mixed and heat denatured to provide the primers for PCR amplification to synthesize a longer fragment that joined segments 1 and 2. The longer fragment was heat denatured and PCR amplified with oligo #7 (containing the *Eco*RI restriction site) and oligo #8 (containing the *Pst*I site). The PCR amplified fragment was restricted with *Eco*RI and *Pst*I and sub-cloned into pKK223-3, where the fusion is constitutively expressed. (**B**) Pairs of oligonucleotides used to construct native Spinach and Spinach tRNA genes. The RNA species on the left column were constructed by using different combination of oligonucleotides as described.

Supplementary Figure S2. Spinach-tRNA fusions. Sequence and secondary structure of the fusion of the Spinach aptamer (in green) into the following domains of *Ec* tRNA^{Tyr2}: (**A**) the V-loop, (**B**) the D-loop, (**C**) the T-loop, and (**D**) the V-loop with U71 and U72 substitutions in the acceptor stem, resulting in the G1-U72 and G2-U71 base pairs, respectively. Notably, the U71-U72 variant in (**D**) has the potential to re-arrange the AU^{71}UACCA^{76} sequence (in yellow) such that the AU^{71}U^{72} nucleotides are bulged out from the acceptor stem and the remaining nucleotides A^{73}CCA^{76} are paired to the acceptor stem. This alternative structure is unstable in *E. coli* (64). The secondary structure of the Spinach aptamer was drawn based on its crystal structures (17,29), where the blue star indicates the bound ligand DFHBI. Each fusion is shown with the aptamer and the tRNA in two stand-alone structures without mutual interactions.

Supplementary Figure S3. Imaging Spinach fusion with tRNA$^{Leu/CAG}$, tRNA$^{Ala/UGC}$, tRNA$^{Phe/GAA}$, and tRNA$^{Pro/UGG}$ in *E. coli*. (**A**) Insertion of Spinach (in green) to the V-loop of *E. coli* tRNA$^{Leu/CAG}$. The secondary structure of the Spinach motif was drawn based on crystal structures of the aptamer (17,29), where the blue star indicates the bound ligand DFHBI. (**B**) Fluorescence microscope image of *E. coli* JM109 cells expressing V-Spinach tRNA$^{Leu/CAG}$. (**C**) Sequence and secondary structure of the V-Baby-Spinach fusion to *E. coli* tRNA$^{Ala/UGC}$, tRNA$^{Phe/GAA}$, and tRNA$^{Pro/UGG}$. (**D**) Fluorescence microscope images of *E. coli* JM109 cells expressing V-Baby-Spinach fusion for tRNA$^{Ala/UGC}$, tRNA$^{Phe/GAA}$, and tRNA$^{Pro/UGG}$. DIC images and scale bars are shown for (**B**) and (**D**). The scale bars represent 2 μm.

Supplementary Figure S4. Northern analysis. (**A**) Total RNA isolated from *E. coli* cells expressing Spinach fusions of tRNA^{Tyr} was probed with two DNA oligonucleotides, with one targeting the Spinach motif and the second targeting the 5S rRNA. The absence of detection of the Spinach alone construct indicates that this aptamer by itself was unstable in *E. coli*. (**B**) Total RNA isolated as above was probed with two DNA oligonucleotides, with one targeting the 5S rRNA and the second targeting a unique sequence in the native *Ec* tRNA^{Tyr2}. The migration positions of Spinach tRNA (183 nucleotides), 5S rRNA (120 nucleotides), and *Ec* tRNA^{Tyr2} (85 nucleotides) are indicated with arrows. In (**A**) and (**B**),

Supplementary Figure S5. Imaging V-Spinach tRNA^{Tyr} with the amber-reading 5'-CUA anticodon. (**A**) Fluorescence microscope image of *E. coli* JM109 cells expressing V-Spinach tRNA^{Tyr} upon induction with IPTG (left) relative to a no-IPTG control (right). The Spinach-tRNA fusion was induced by IPTG for 30, 60 or 90 min, and cells were washed and incubated with DFHBI on a slide glass for fluorescent imaging. Top panel is the image from a bright field, whereas the middle panel is the fluorescence image excited by 488 nm Argon ion laser, and the lower panel shows the merged image. Images of 60-min induction are shown as a representative.

The scale bar represents 2 μm. (**B**) Quantification of fluorescence over the IPTG (1 mM)-induced time course of *E. coli* JM109 cells. Average intensity of the pixels corresponding to the area of a single *E. coli* cell was quantified by image analysis software as in Figure 1B. Error bars are expressed as SEM, where n = 100 for each of the time points.

Supplementary Figure S6. **Analysis of Spinach tRNA^{Tyr} activity for protein synthesis**. (**A**) Aminoacylation by *E. coli* TyrRS and synthesis of Tyr-tRNA^{Tyr} as a function of tRNA concentration. Error bars represent standard deviations (n = 3). (**B**) Determination of the k_{off} of charged tRNA from the ternary complex with EF-Tu-GTP. Fractions of the remaining ternary complex as a function of time were monitored over time. Reactions were conducted in an ice bath and contained 1.0 μM EF-Tu and 40 nM Tyr-tRNA^{Tyr} (3'-end labeled with ^{32}P) in 0.5 M NH_4Cl, 20 mM $MgCl_2$, 20 uM GTP, 5 mM DTT, 1.2 mM phosphoenolpyruvate, 50 μg/mL pyruvate kinase, and 50 mM Hepes pH 7.0. Errors are SDs (n = 3). Data points were plotted on a semi-log scale to the equation y = mx + b, where m is the slope, representing the rate constant. (**C**) Kinetics of accommodation of tRNA to the ribosome upon GTP hydrolysis. Time courses of GTP hydrolysis upon initial selection of tRNA to the A-site of an *E. coli* ribosome were monitored. Errors are SDs, n = 3. (**D**) Kinetics of dipeptide fMY synthesis. Time courses of the dipeptide synthesis were monitored. Data for GTP hydrolysis and peptide bond formation were fit to the equation $y = y_0 + A(1-e^{-k_{obs}t})$, where y_0 is the y intercept, A is a scaling constant, k_{obs} is the observed rate constant and *t* is time in second. Each data point was the average of 3 independent experiments, showing SD for each (n = 3). For curve fitting in A to D, data points were fit to the appropriate equation, giving rise to the rate constant in each experiment and SEs from curve fitting.