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Supplemental information

Development of mammalian cell logic gates controlled by unnatural amino acids

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1. Supplementary Figures

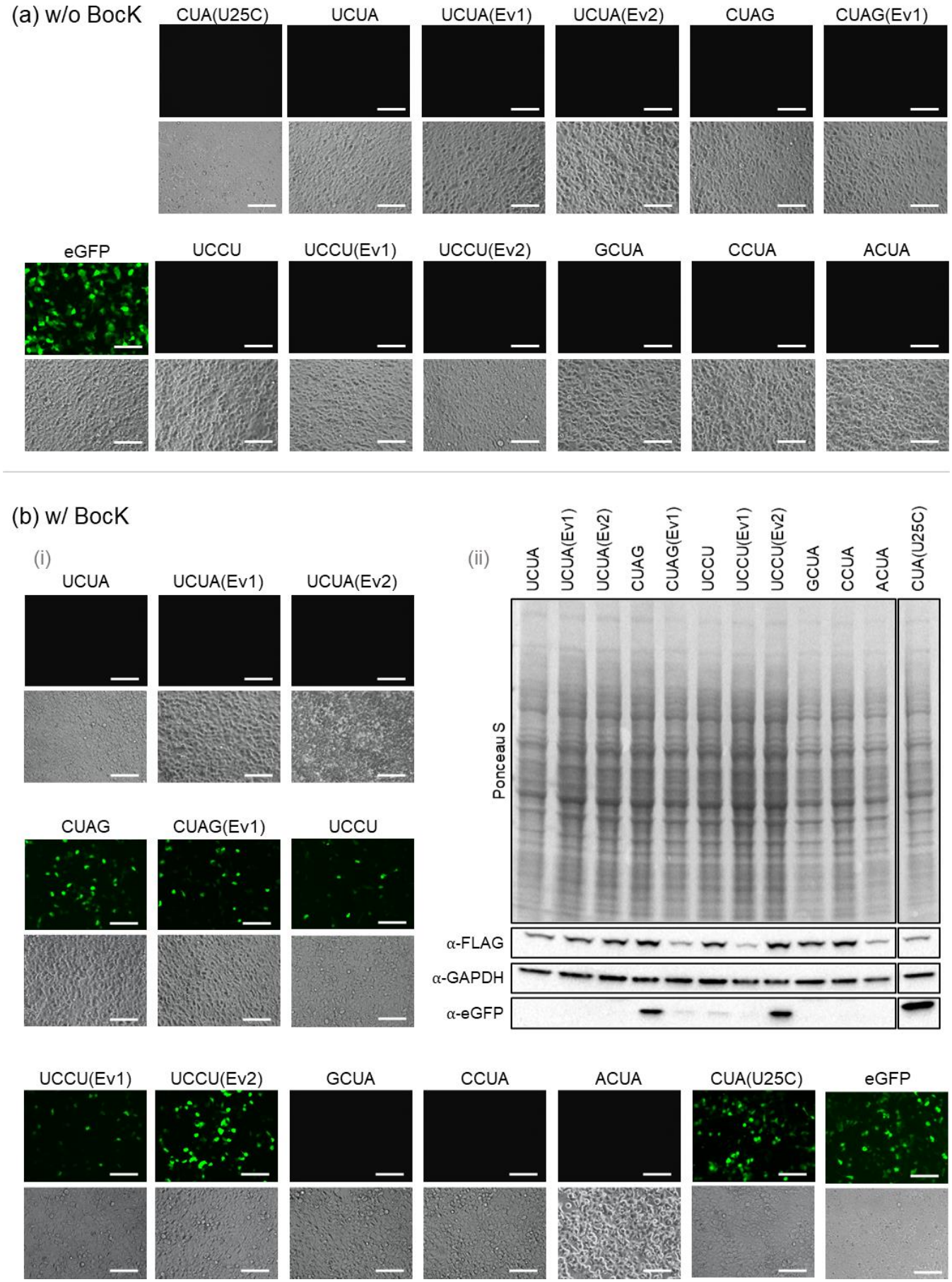

Figure S1. Production of eGFP(150XXXX) using different Pyl tRNA variants. Related to Figure 2. HEK293 cells were transiently transfected with the designated evaluation vector encoding the wild-type *M. mazei* PylRS, a Pyl tRNA variant and an eGFP reporter with the appropriate quadruplet codon at the position for the 150th amino acid residue in the absence (**a**) or presence (**b**) of 1 mM BocK for 24 h. No GFP fluorescence was detected for any of the variants when BocK was not supplemented. Transfection with a vector carrying the wild-type eGFP was used as the positive control. Bright field images show cell confluency in each condition. All images are 223 µm × 167 µm, scale bars denote 50 µm. Immunoblotting with α-FLAG and α-GFP antibodies were used to detect PylRS and full-length eGFP, respectively. An irrelevant lane was digitally deleted from the blot and is indicated by the gap between ACUA and CUA(U25C).

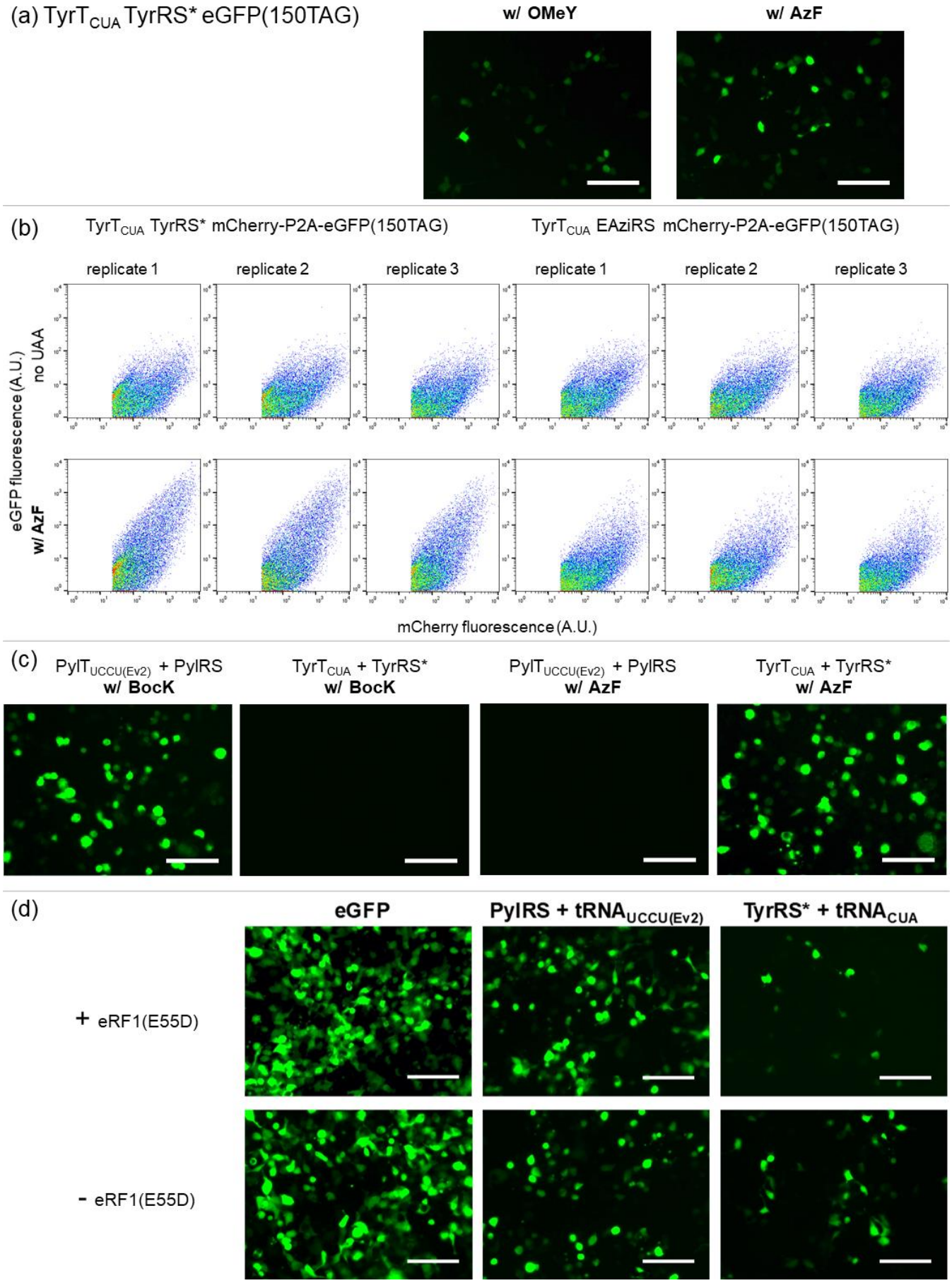

Figure S2. Utilisation of TyrRS*/tRNA$_{CUA}$ as the second orthogonal pair. Related to Figure 3. (**a**) Incorporation of OMeY and AzF into eGFP(150TAG) with TyrRS*. Fluorescence microscopy images of OMeY and AzF incorporation into the 150th amino acid position of eGFP demonstrate higher levels of eGFP fluorescence when AzF is incorporated. (**b**) Flow cytometry analysis of incorporation of AzF into eGFP(150TAG) by TyrRS* or EAziRS. HEK293 cells transfected with TyrT$_{CUA}$ TyrRS* mCherry-P2A-eGFP(150TAG) or TyrT$_{CUA}$ EAziRS mCherry-P2A-eGFP(150TAG) were incubated in the absence or presence of AzF for 48 hours before flow cytometry analysis. Events were gated for HEK293 cells and to exclude doublets. Cells were then gated to include only transfected (i.e. mCherry positive) cells. Fluorescence intensities are given in arbitrary units (A.U.). Three biological replicates for each condition are shown. The mean fluorescence intensities were used to calculate the incorporation efficiency as per the equation in Figure 2. The incorporation efficiency is 8% ± 2% for TyrT$_{CUA}$ TyrRS* mCherry-P2A-eGFP(150TAG), and 0.6% ± 0.3% for TyrT$_{CUA}$ EAziRS mCherry-P2A-eGFP(150TAG). (**c**) Orthogonality of tRNA/aaRA pairs. HEK293 cells transfected with PylRS/tRNA$_{UCCU(Ev2)}$ or TyrRS*/tRNA$_{CUA}$ reporter vector and incubated for 24 hours in the presence of AzF or BocK, respectively. Fluorescent eGFP was only

detected when the aaRS/tRNA pairs were in the presence of their cognate unnatural amino acid depicting mutual orthogonality of the pairs. (**d**) Impact of eRF1(E55D) on incorporation efficiency of TyrRS*/tRNA$_{CUA}$ or PylRS/tRNA$_{UCCU(Ev2)}$. No substantial increase in eGFP fluorescence was observed. eRF1(E55D) was also expressed alongside wildtype eGFP as a control. All fluorescent images are 223 µm × 167 µm, scale bars denote 50 µm.

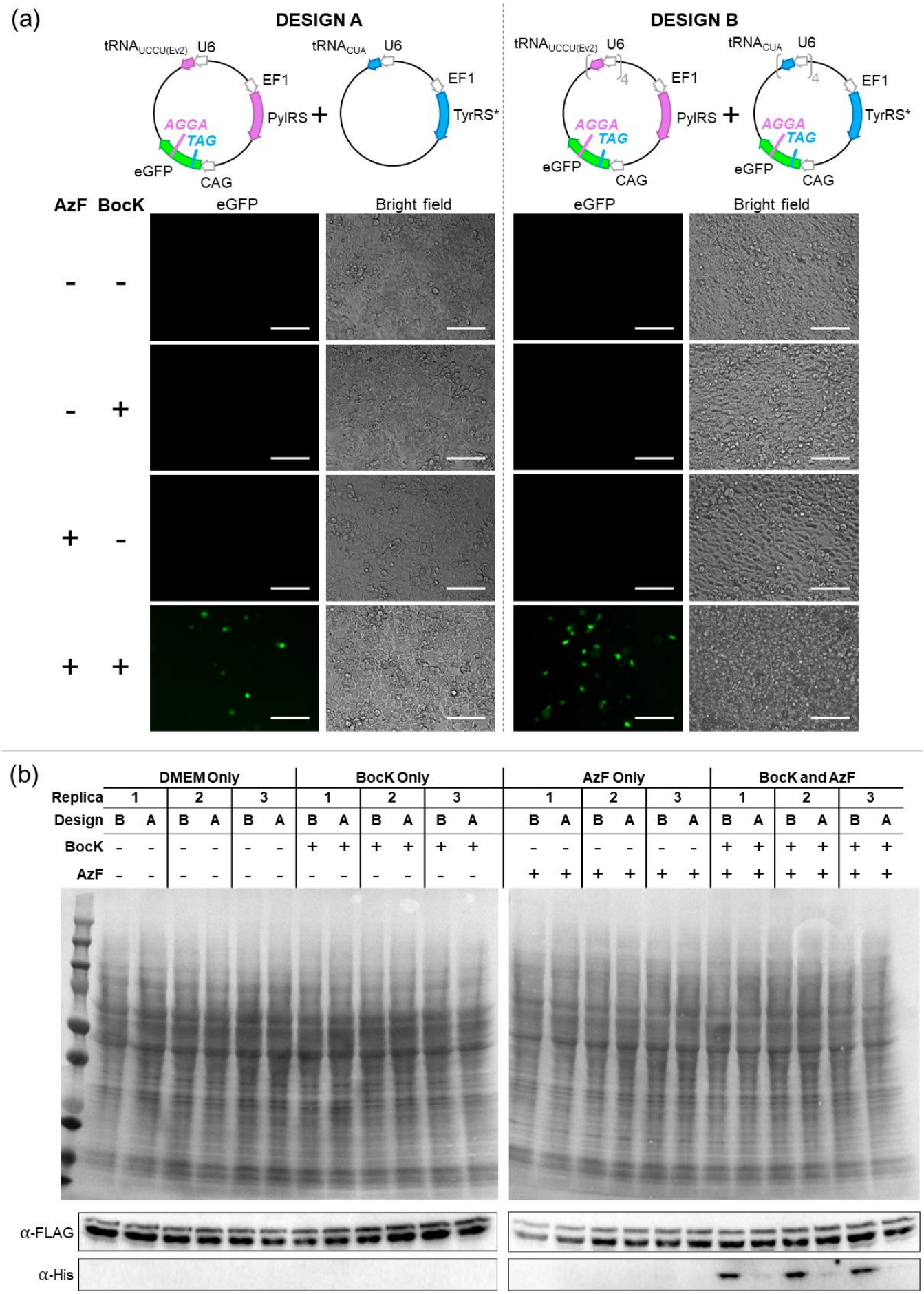

Figure S3. Incorporation of AzF and BocK into a single eGFP using Tyr tRNA$_{CUA}$ and Pyl tRNA$_{UCCU(Ev2)}$. Related to STAR Methods. (**a**) AzF was incorporated at the 40th residue of the mutant eGFP using amber suppression and BocK was incorporated into the 150th residue of the eGFP mutant using AGGA suppression. Double incorporation was investigated with two experimental designs: Design A comprised of a single reporter gene and tRNA for respective pairs, Design B an attempt at optimisation with increased tRNA and reporter copy number. eGFP fluorescence was only detected when cells were supplemented with both AzF and

BocK, Design B demonstrated higher levels of eGFP fluorescence. (**b**) Western blot of Design A vs Design B with three biological replicates of 4 conditions: (1) no unnatural amino acid, (2) BocK only, (3) AzF only and (4) BocK and AzF. Full length eGFP was only detected when both unnatural amino acids are supplemented, with Design B yielding substantially higher eGFP band intensity. All fluorescent images are 223 µm × 167 µm, scale bars denote 50 µm.

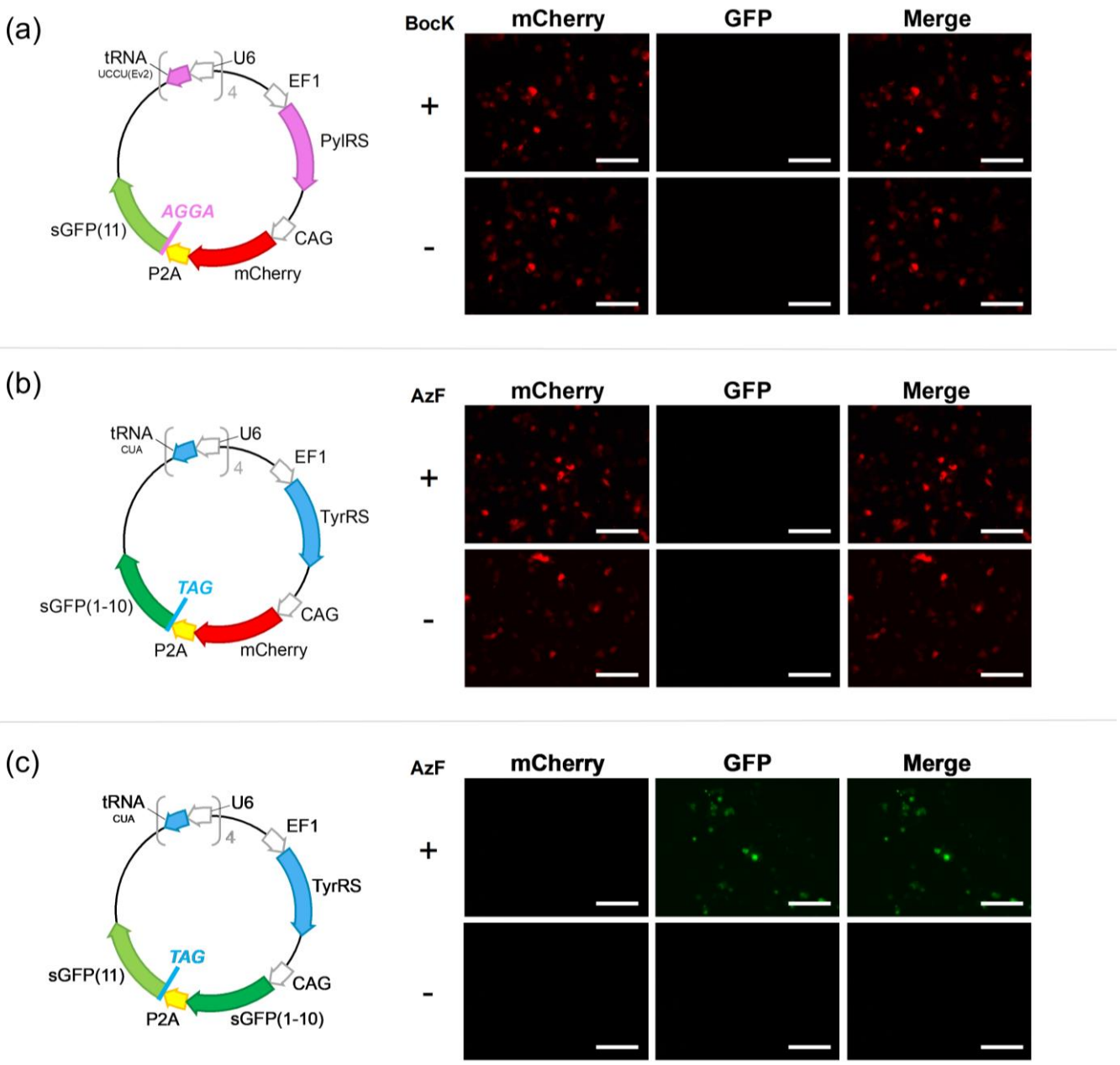

Figure S4. Logic gate vector specificity test. Related to Figure 4. (**a**-**c**) Individual transfection of logic gate vectors in the presence (+) or absence (-) of their respective unnatural amino acid (BocK or AzF) at 1 mM. All images depicted are 223 µm × 167 µm, scale bars denote 50 µm.

2. Methods S1

2.1 Table S1. Primers used in this study. Related to STAR methods.

Primer ID	Primer sequence (5'-3')
Primer 1	GGGGGGATACGGGGAAAAGGCCTAAAAACCGCACTTGTCCGGAAAC
Primer 2	GGTCAGGGCTGGGGCAGAGGCTTGCACAGGGGCAGACATG
Primer 3	ATCATGGCTGATAAGCAGAAGAAC
Primer 4	CGTTCTTCTGCTTATCAGCCATGATATAGACTCTAGTGGCTGTTGTAGTTGTACTCCAGCTTG
Primer 5	CGTTCTTCTGCTTATCAGCCATGATATAGACCTAGGTGGCTGTTGTAGTTGTACTCCAGCTTG
Primer 6	CGTTCTTCTGCTTATCAGCCATGATATAGACTCCTGTGGCTGTTGTAGTTGTACTCCAGCTTG
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Primer 19	ACCTGATCATGTAGATCGAATGGACTACTAAATCCGTTCAGCCGGGTTAGATTCC
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Primer 21	ACCTGATCATGTAGATCGAATGGACTCCTAAATCCGTTCAGCCGGGTTAGATTCC
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Primer 23	ACCGGAGCGATCGCAACCGGTCGGGCAGGAAGAGGGCCTATTTCCCAT
Primer 24	ACTTACGCTTGCCACCATGGCTAGCGACTACAAGGACGACGACGACAAGGCAAGCAGTAACTTGATTAAACAATTGCAAGAG
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Primer 31	AGTGCGGTTTTTCTTAAGAGGTTGGGCAGGAAGAGGGCCTATTTC
Primer 32	GGATACGGGGAAAAGGAGGCCTACATGC
Primer 33	GTAAACGGCCACAAGTTCGTCGATTGGGCAGGAAGAGGGCCTATTTC
Primer 34	TCGACGAACTTGTGGCCGTTTACCATGCAAATATTAAAAAATGGTGGGGGAAG
Primer 35	CTGGTGGAGAACTTGCCGAATTGGGCAGGAAGAGGGCCTATTTC

Primer 36	AATTCGGCAAGTTCTCCACCAGACATGCAAATATTAAAAAATGGTGGGGGAAG
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Primer 38	AGGGTCAGCTTGCCCTAGGTGGCATCG
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Primer 45	TTTTTGGCAGAGGGAAA AAGATCTCACATG
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Primer 48	TGTCTCATCATTTTGGCAAAGAATTCATGTCCAAAGGAGAAGAACTGTTTACC
Primer 49	TTCTCTTCCACGTCGCCAGCCTGTTTCAGCAGGCTGAAATTGGTGGCGCCTCCAGACGCTTTTTCATTTGGATCTTTGCTCAGGACTGTTTG
Primer 50	AGCAAGGGCGAGGAGCTG
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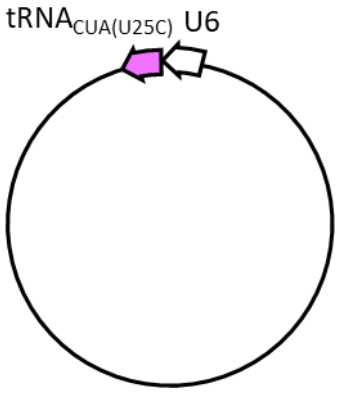
Primer 53	AACAGCTCCTCGCCCTTGCTCACCGACTTTTGTCCAGGTCCAGG
Primer 54	TGCCGCCAGAACACAGCTGAAGC
Primer 55	GAGTGCGATCGGGCCTTGC
Primer 56	CGCAAGGCCCGATCGCACTCCTGTGTGGCTTCGATCCTACCGCTGAC
Primer 57	ACTATAACCCTGCAGCAGGTTG
Primer 58	ACCTGCTGCAGGGTTATAGTGCCGCCTGTGCCAACAAACAGTACGGTGTGGTGC
Primer 59	TCCACCACACTGGACTAGTGGATCCTTATCATTAAACGGGCCCTTTCCAGC

2.2 Working vector sequences. Related to STAR methods.

Vector 1 has kanamycin resistance; all other vectors have ampicillin resistance. Gene sequences are colour-coded using the following: U6 promotor, **PylT** (anticodon highlighted), EF1 promotor, PylRS, CAG promotor and chimeric intron, **eGFP** (150th codon highlighted), **TyrT** (anticodon highlighted), **TyrRS**, **mCherry**, **linker**, **P2A**, **sGFP(1-10)**, **sGFP(11)7x** (AGGA/TAG codon highlighted)

2.2.1 Vector 1: Pyl tRNACUA(U25C)



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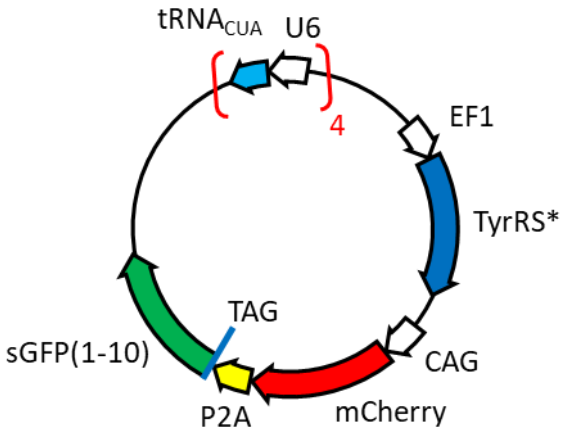
2.2.2 Vector 55: 4x(Tyr tRNA$_{CUA}$) TyrRS* mCherry-P2A-TAG-sGFP(1-10)



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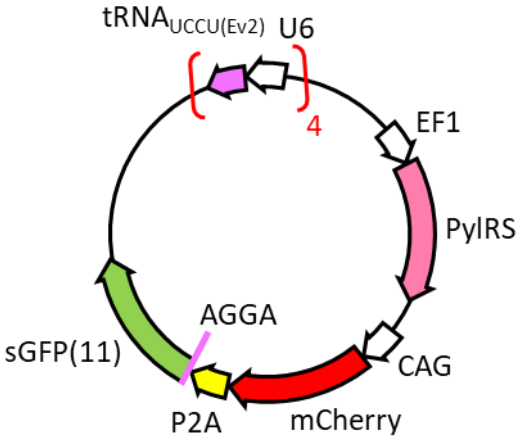

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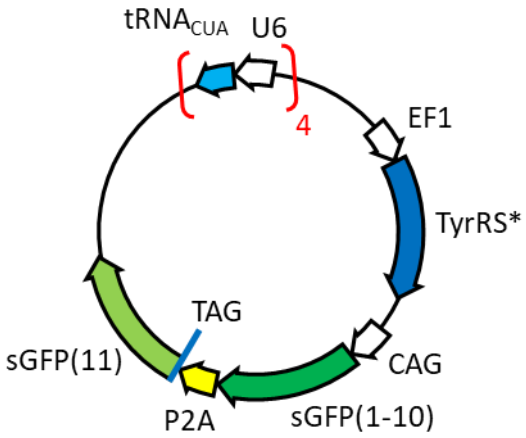
2.2.4 Vector 57: 4x(Tyr tRNA$_{CUA}$) TyrRS* sGFP(1-10)-TAG-sGFP(11)



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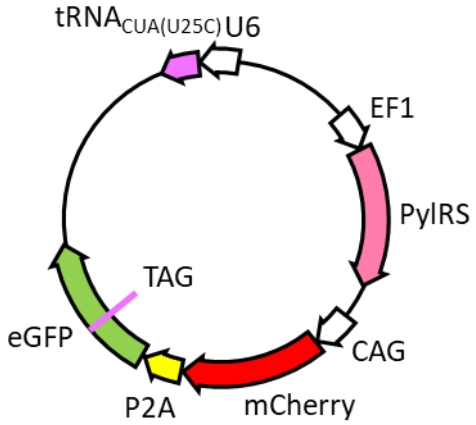
2.2.5 Vector 58: Pyl tRNA$_{CUA(U25C)}$ PylRS mCherry-P2A-eGFP(150TAG)



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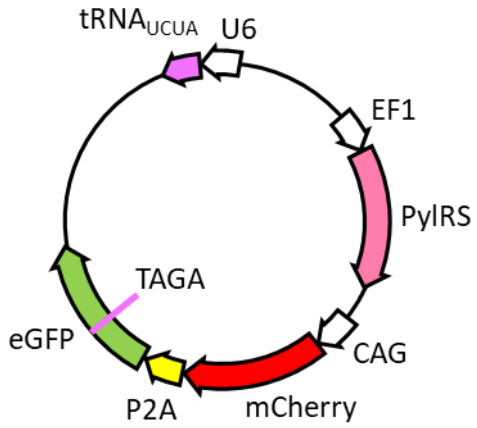
2.2.6 Vector 59: Pyl tRNA$_{UCUA}$ PylRS mCherry-P2A-eGFP(150TAGA)



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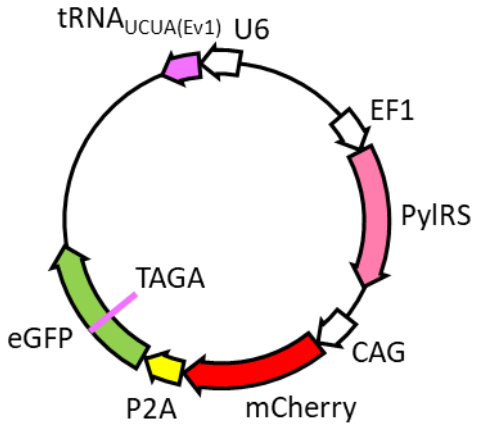
2.2.7 Vector 60: Pyl tRNA$_{UCUA(Ev1)}$ PylRS mCherry-P2A-eGFP(150TAGA)



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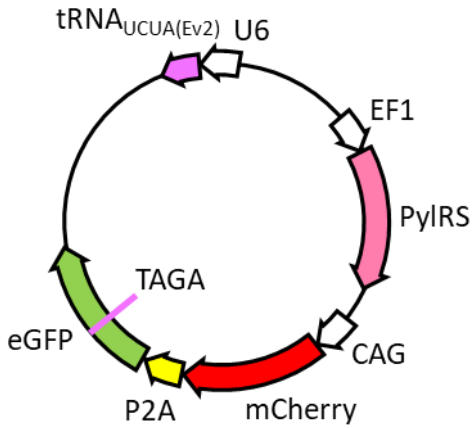
2.2.8 Vector 61: Pyl tRNA$_{UCUA(Ev2)}$ PylRS mCherry-P2A-eGFP(150TAGA)



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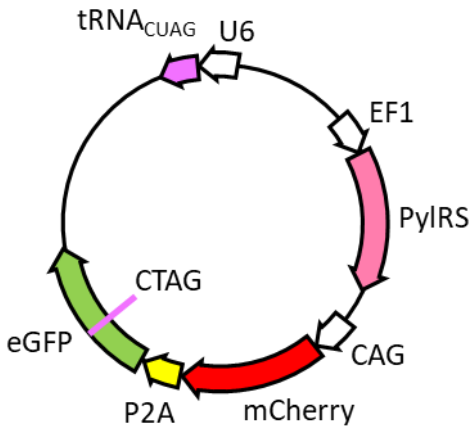
2.2.9 Vector 62: Pyl tRNA$_{CUAG}$ PylRS mCherry-P2A-eGFP(150CTAG)



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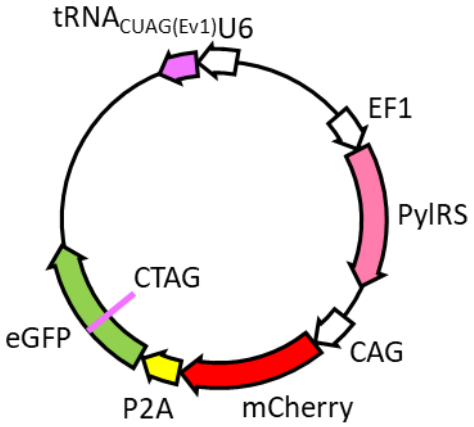
2.2.10 Vector 63: Pyl tRNA$_{CUAG(Ev1)}$ PylRS mCherry-P2A-eGFP(150CTAG)



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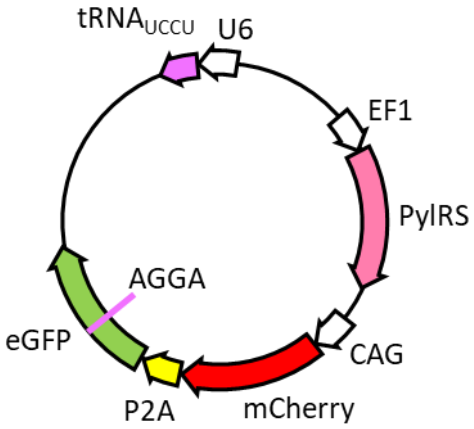
2.2.11 Vector 64: Pyl tRNA$_{UCCU}$ PylRS mCherry-P2A-eGFP(150AGGA)



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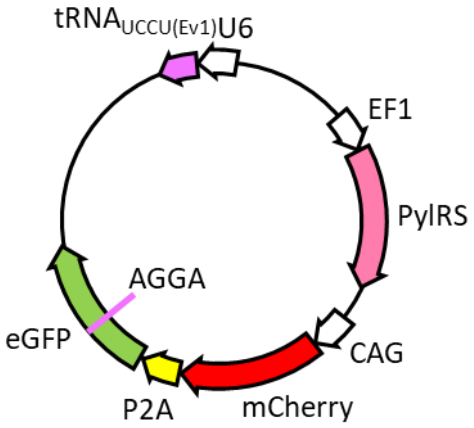
2.2.12 Vector 65: Pyl tRNA$_{UCCU(Ev1)}$ PylRS mCherry-P2A-eGFP(150AGGA)



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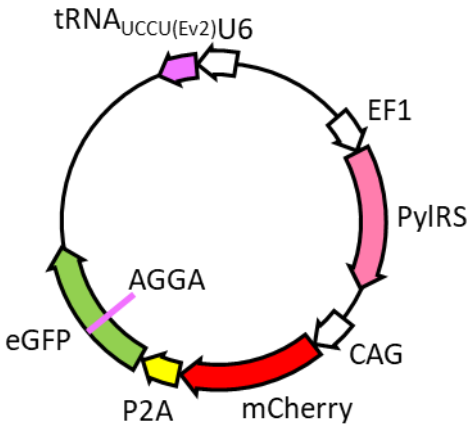
2.2.13 Vector 66: Pyl tRNA$_{UCCU(Ev2)}$ PylRS mCherry-P2A-eGFP(150AGGA)



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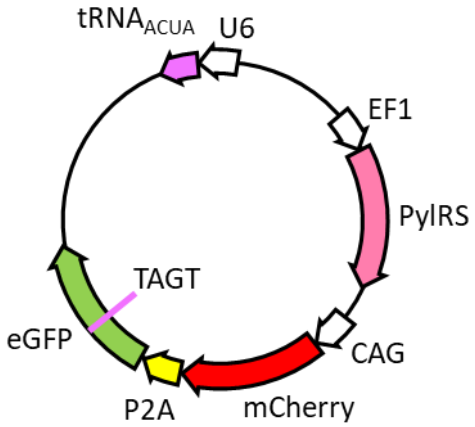
2.2.14 Vector 67: Pyl tRNA$_{ACUA}$ PylRS mCherry-P2A-eGFP(150TAGT)



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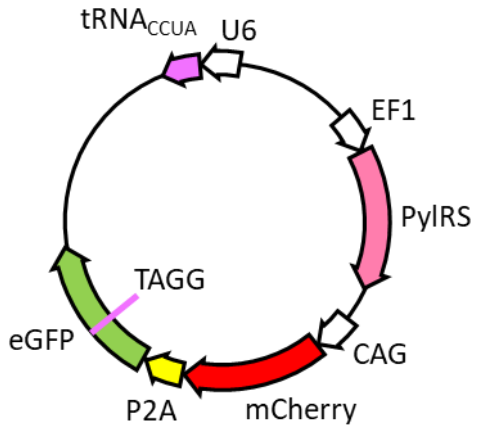
2.2.15 Vector 68: Pyl tRNA$_{CCUA}$ PylRS mCherry-P2A-eGFP(150TAGG)



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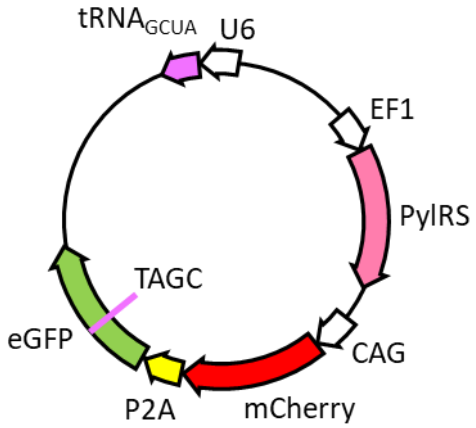
2.2.16 Vector 69: Pyl $tRNA_{GCUA}$ PylRS mCherry-P2A-eGFP(150TAGC)



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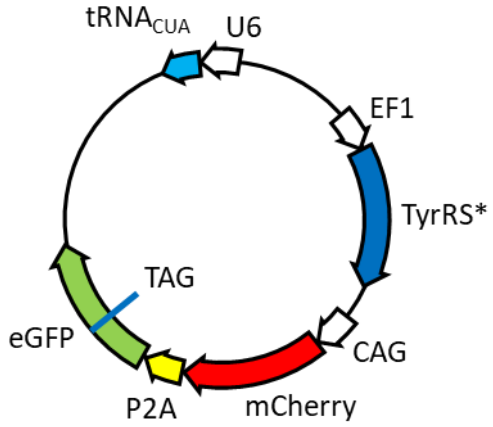
2.2.17 Vector 71: Tyr tRNA$_{CUA}$ TyrRS* mCherry-P2A-eGFP(150TAG)



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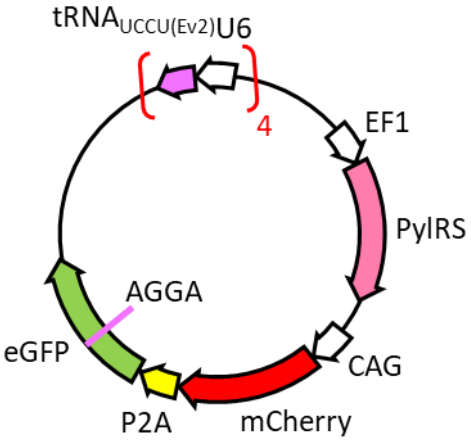
2.2.18 Vector 73: 4x(Pyl tRNA$_{UCCU(Ev2)}$) PylRS mCherry-P2A-eGFP(150AGGA)



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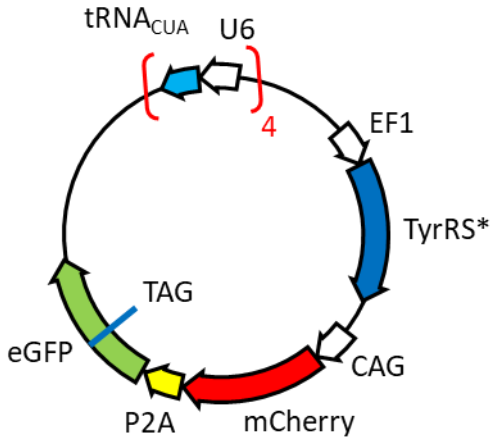
2.2.19 Vector 74: 4x(Tyr $tRNA_{CUA}$) TyrRS* mCherry-P2A-eGFP(150TAG)



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