

Cell Reports Methods, Volume 1

Supplemental information

**Development of mammalian cell logic gates
controlled by unnatural amino acids**

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1. Supplementary Figures

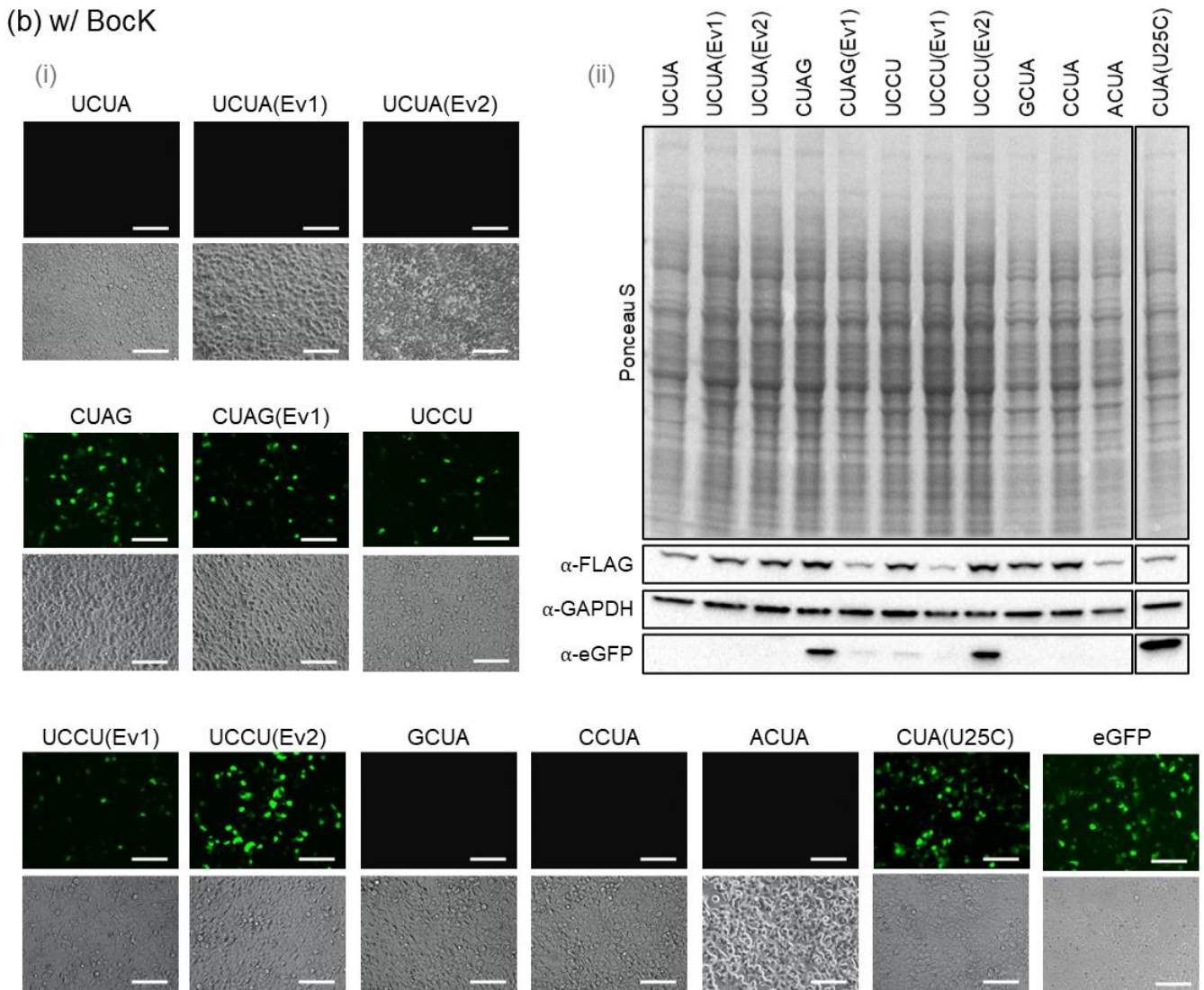
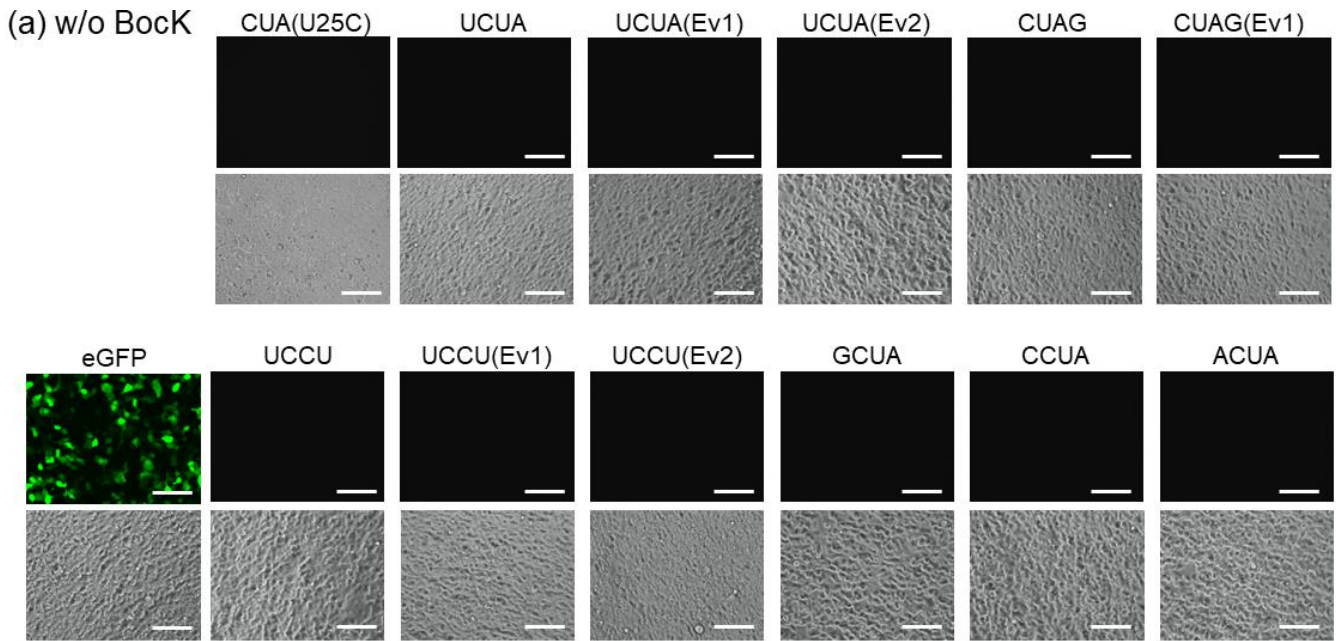


Figure S1. Production of eGFP(150XXXX) using different Pyl tRNA variants. Related to Figure 2. HEK293 cells were transiently transfected with the designated evaluation vector encoding the wild-type *M. mazei* PylRS, a Pyl tRNA variant and an eGFP reporter with the appropriate quadruplet codon at the 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 \times 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).

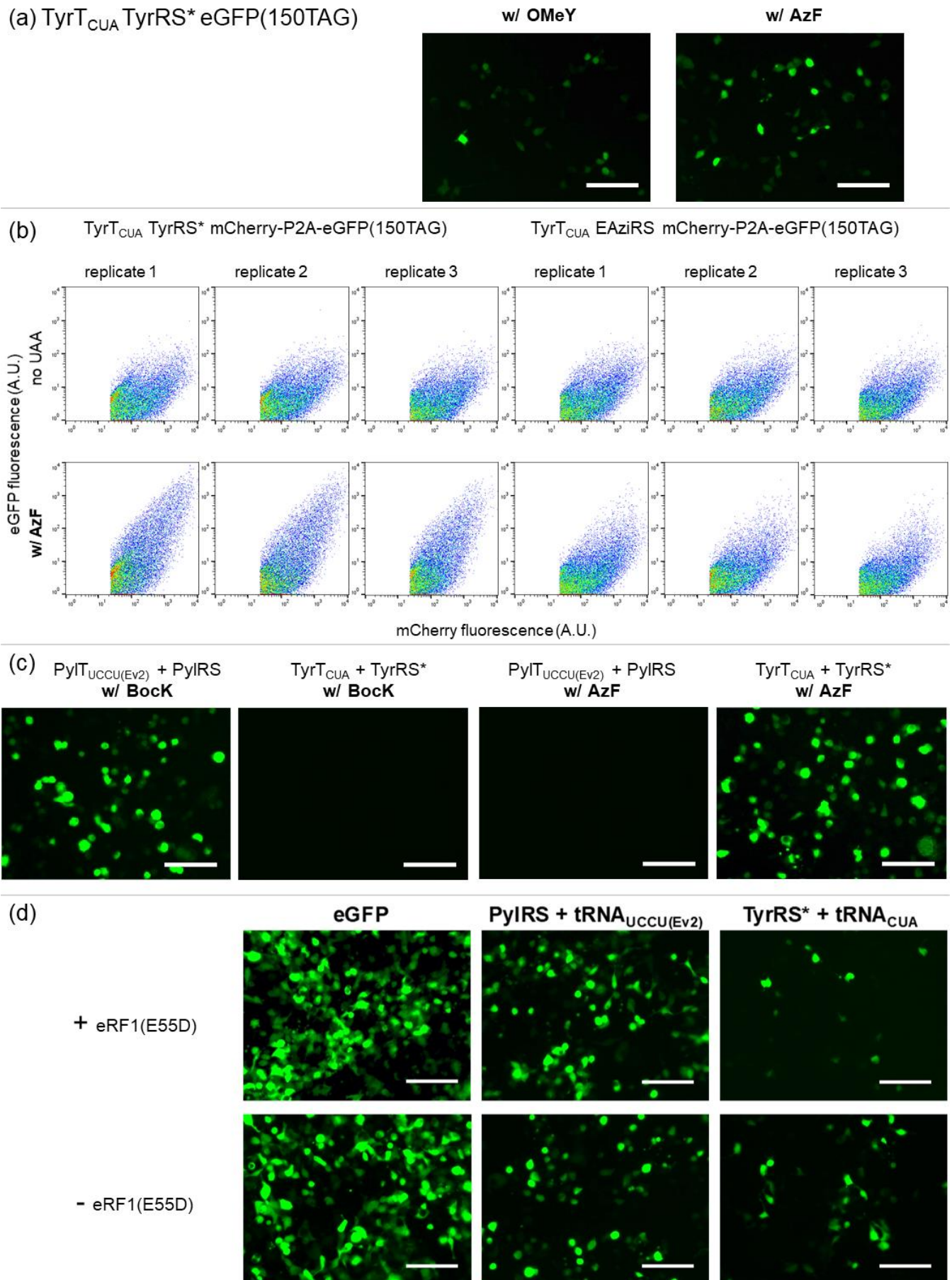


Figure S2. Utilisation of TyrRS*/tRNA_{CUA} as the second orthogonal pair. Related to Figure 3. (a) Incorporation of OMeY and AzF into eGFP(150TAG) with TyrRS*. Fluorescence microscopy images of OMeY and AzF incorporation into the 150th amino acid position of eGFP demonstrate higher levels of eGFP fluorescence 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 PyIRS/tRNA_{UCCU(EV2)} or TyrRS*/tRNA_{CUA} reporter vector and incubated for 24 hours in the presence of AzF or BockK, 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.

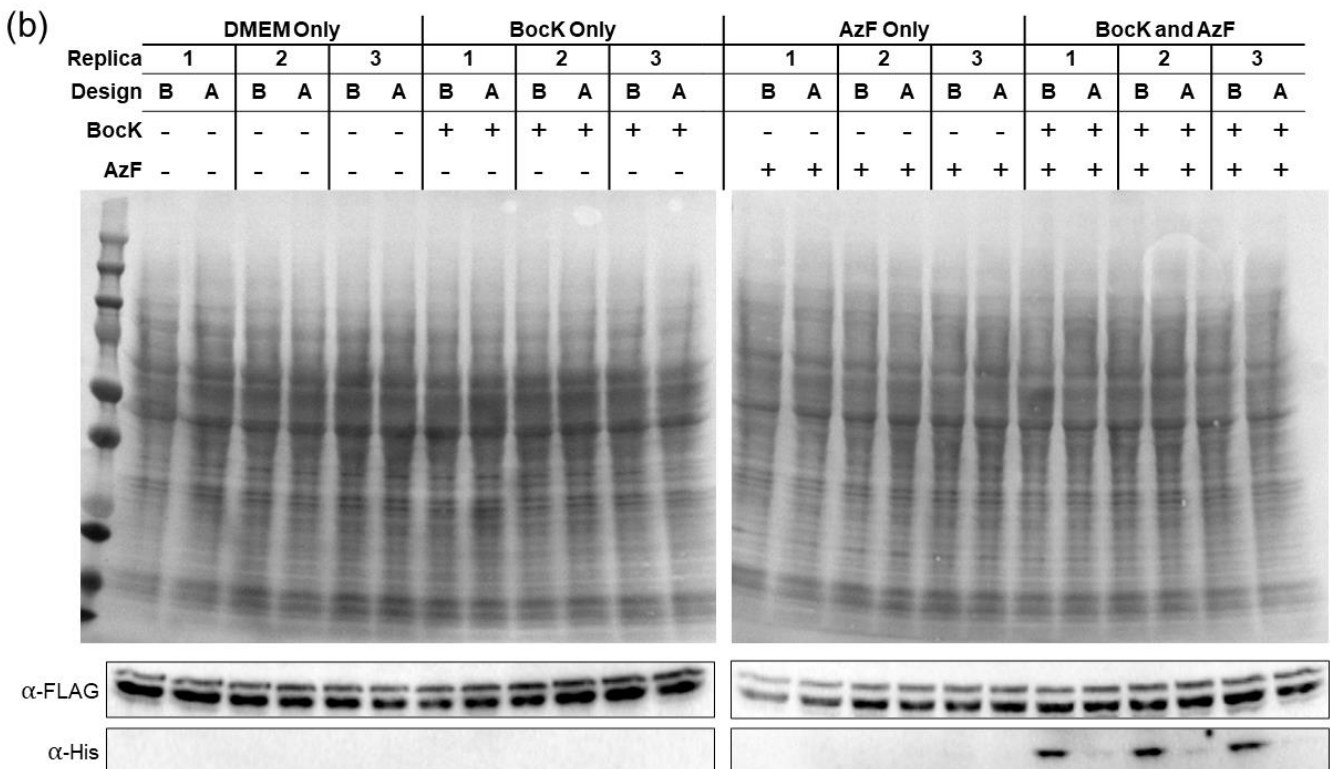
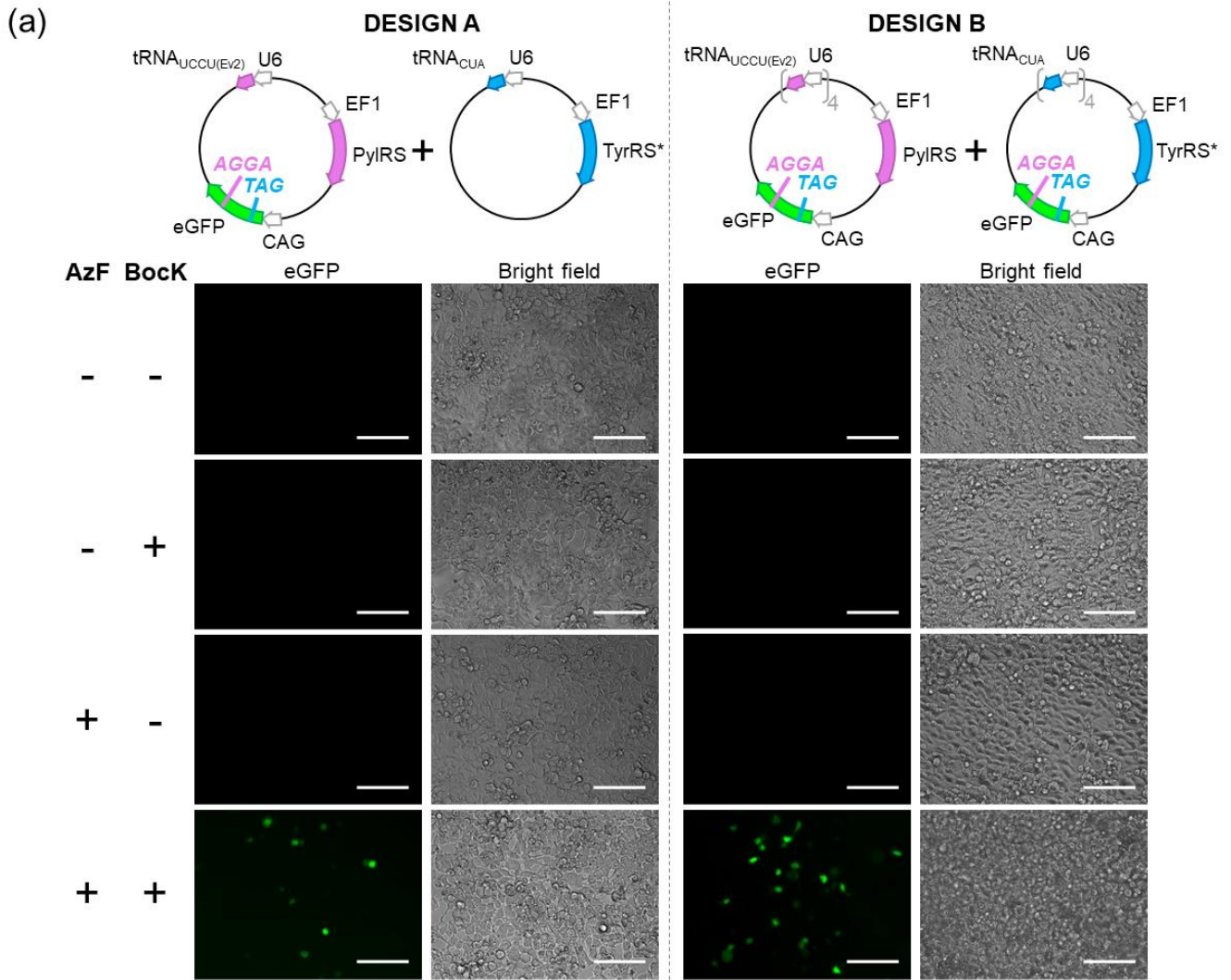


Figure S3. Incorporation of AzF and BocK into a single eGFP using Tyr tRNA_{CUA} and Pyl tRNA_{UCCU(EV2)}. Related to STAR Methods. (a) AzF was incorporated at the 40th residue of the mutant eGFP using amber suppression and BocK was incorporated into the 150th residue of the eGFP mutant using AGGA suppression. 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 $\mu\text{m} \times 167 \mu\text{m}$, scale bars denote 50 μm .

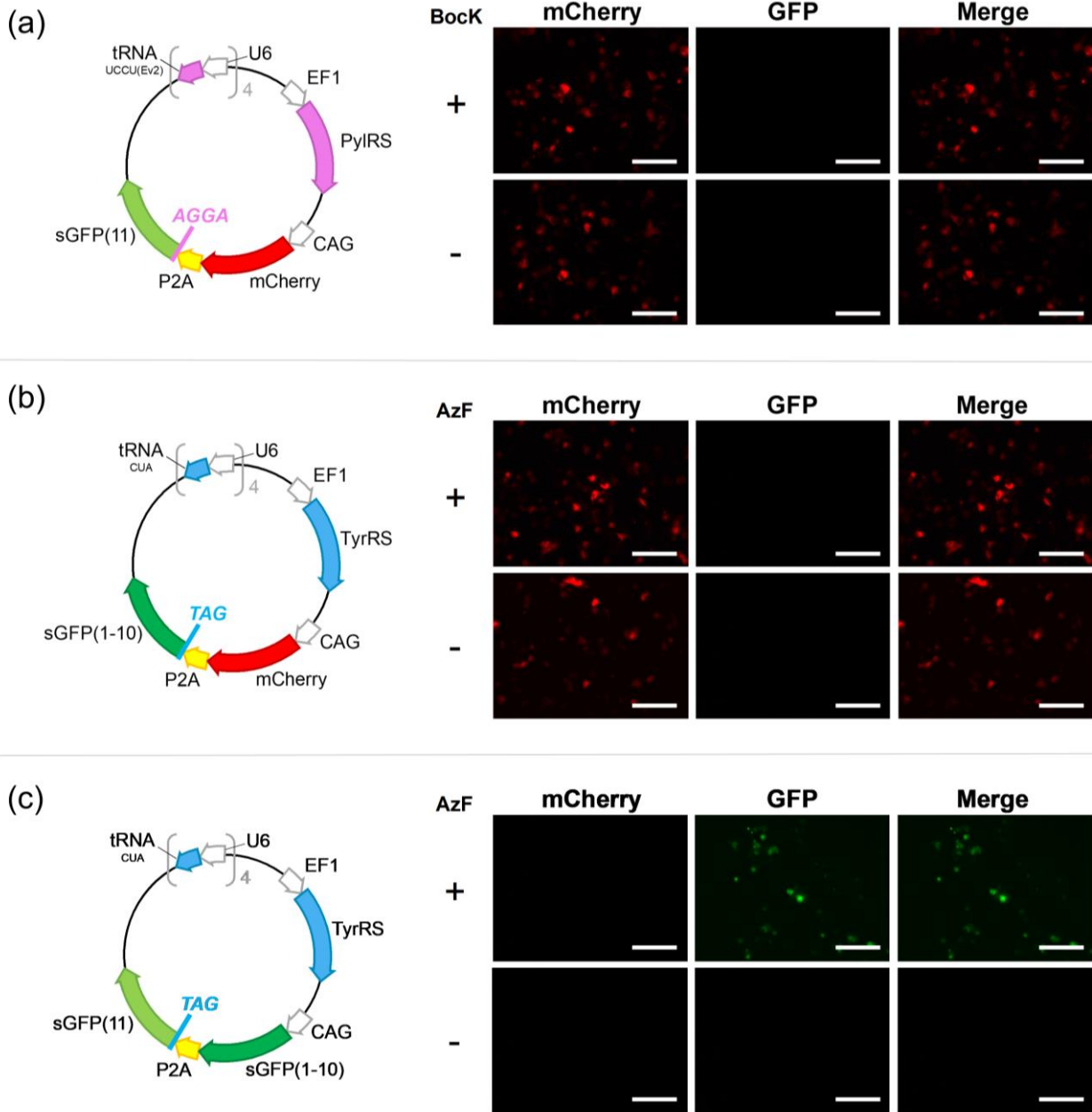


Figure S4. Logic gate vector specificity test. Related to Figure 4. (a-c) Individual transfection of logic gate vectors in the presence (+) or absence (-) of their respective unnatural amino acid (BocK or AzF) at 1 mM. All images depicted are 223 $\mu\text{m} \times 167 \mu\text{m}$, scale bars denote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

2. Methods S1

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

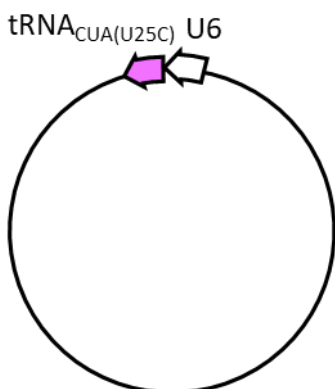
Primer ID	Primer sequence (5'-3')
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Primer 2	GGTCAGGGCTGGGGCAGAGGCTTGCACAGGGGCAGACATG
Primer 3	ATCATGGCTGATAAGCAGAAGAAC
Primer 4	CGTTCTTCTGCTTATCAGCCATGATATAGACTCTAGTGGCTGTTGTAGTTGACTCCAGCTTG
Primer 5	CGTTCTTCTGCTTATCAGCCATGATATAGACTCTAGTGGCTGTTGTAGTTGACTCCAGCTTG
Primer 6	CGTTCTTCTGCTTATCAGCCATGATATAGACTCTGTGGCTGTTGTAGTTGACTCCAGCTTG
Primer 7	CGTTCTTCTGCTTATCAGCCATGATATAGACTCTAGTGGCTGTTGTAGTTGACTCCAGCTTG
Primer 8	CGTTCTTCTGCTTATCAGCCATGATATAGACTCTAGTGGCTGTTGTAGTTGACTCCAGCTTG
Primer 9	CGTTCTTCTGCTTATCAGCCATGATATAGACTCTAGTGGCTGTTGTAGTTGACTCCAGCTTG
Primer 10	TTCGATCTACATGATCAGGTTTCCGGTG
Primer 11	ACCTGATCATGTAGATCGAATGGACTTCTAAATCCGTTCCAGCCGGGTTAGATTCC
Primer 12	ACCTGATCATGTAGATCGAATGGGCTTCTAATCTCGTTCCAGCCGGGTTAGATTCC
Primer 13	ACCTGATCATGTAGATCGAATGGGCTTCTAATCCGTTCCAGCCGGGTTAGATTCC
Primer 14	ACCTGATCATGTAGATCGAATGGACTCTAGAATCCGTTCCAGCCGGGTTAGATTCC
Primer 15	ACCTGATCATGTAGATCGAATGGCCTCTAGAATCCGTTCCAGCCGGGTTAGATTCC
Primer 16	ACCTGATCATGTAGATCGAATGGACTTCTAATCCGTTCCAGCCGGGTTAGATTCC
Primer 17	ACCTGATCATGTAGATCGAATCCTCTTCTAATAGTTCCAGCCGGGTTAGATTCC
Primer 18	ACCTGATCATGTAGATCGAAGGGGCTTCTATCCGTTCCAGCCGGGTTAGATTCC
Primer 19	ACCTGATCATGTAGATCGAATGGACTACTAAATCCGTTCCAGCCGGGTTAGATTCC
Primer 20	ACCTGATCATGTAGATCGAATGGACTGCTAAATCCGTTCCAGCCGGGTTAGATTCC
Primer 21	ACCTGATCATGTAGATCGAATGGACTCCTAAATCCGTTCCAGCCGGGTTAGATTCC
Primer 22	GAAAAGGAGGCTACATGCAAATATTAATAAATGGTGGGGGAAGGATTGCAACCTTC
Primer 23	ACCGGAGCGATCGCAACCGGTCGGGCAGGAAGAGGGCCTATTTCCAT
Primer 24	ACTTACGCTTGCCACCATGGCTAGCGACTACAAGGACGACGACGACAAGGCAAGCAGTAACCTGATTAACAATTGCAAGAG
Primer 25	TCCACCACACTGGACTAGTGGATCCTTATCATTAAACGGGCCCTTCCAGCAAATC
Primer 26	GGGGGATACGGGGAAAAGGCCTCTTAAGAAAACCGCACTTGTC
Primer 27	GTAACGGCCACAAGTTCGTCGATTGGGCAGGAAGAGGGCCTATTTCC
Primer 28	TCGACGAACCTTGTGGCCGTTTACCCTCTTAAGAAAACCGCACTTGTC
Primer 29	CTGGTGGAGAACTTGCCGAATTGGGCAGGAAGAGGGCCTATTTCC
Primer 30	TTCGGCAAGTTCTCCACCAGCCTCTTAAGAAAACCGCACTTGTC
Primer 31	AGTGC GGTTTTTCTTAAGAGGTTGGGCAGGAAGAGGGCCTATTTCC
Primer 32	GGATACGGGGAAAAGGAGGCCTACATGC
Primer 33	GTAACGGCCACAAGTTCGTCGATTGGGCAGGAAGAGGGCCTATTTCC
Primer 34	TCGACGAACCTTGTGGCCGTTTACCATGCAAATATTAATAAATGGTGGGGGAAG
Primer 35	CTGGTGGAGAACTTGCCGAATTGGGCAGGAAGAGGGCCTATTTCC

Primer 36	AATTCGGCAAGTTCTCCACCAGACATGCAAATATTAATAAATGGTGGGGGAAG
Primer 37	TTTTAATATTTGCATGTAGGGGGCAGGAAGAGGGCCTATTC
Primer 38	AGGGTCAGCTTGCCCTAGGTGGCATCG
Primer 39	TCGGCATGGACGAGCTGTACAAGCATCATCACCATCACCCTAAGCTGCCTATCAGAAG
Primer 40	ATGCCACCTAGGGCAAGCTGACCTGAAGTTC
Primer 41	TTGTACAGCTCGTCCATGCCGAGAG
Primer 42	AGAACCTGGACCTGGACAAAAG TCGTAGTCCAAAGGAGAAGAAGCTGTTTACCGGTGTTG
Primer 43	TCCCCATAATTTTTGGCAGAGGGAAAAAGATCTC
Primer 44	GAGAACCCTGGACCTGGACAAAAGTCGAGGACGTGAC CACATGGTCTTCATG
Primer 45	TTTTTGGCAGAGGGAAA AAGATCTCACATG
Primer 46	TTCAGCTGCTGAAACAGGCTGGCGACGTGGAAGAGAACCCTGGACCTGGACAAAAGTCGTAGCGTGACCACATGGTCTTCATG
Primer 47	TTTTTGGCAGAGGGAAAAAGATCTCACATG
Primer 48	TGTCTCATATTTTTGGCAAAGAATTCATGTCCAAAGGAGAAGAAGCTGTTTACC
Primer 49	TTCTCTCCACGTCGCCAGCCTGTTTCAGCAGGCTGAAATGGTGGCGCCTCCAGACGCTTTTTTCATTTGGATCTTTGCTCAGGACTGTTTG
Primer 50	AGCAAGGGCGAGGAGCTG
Primer 51	TGTCCCCATAATTTTTGGCAGAGGGAAAAAGATCTTTACTTGTACAGCTCGTCCATGCCGAGAG
Primer 52	GTCTCATCATTTTTGGCAAAGAATTCGCCACCATGGTGAGCAAGGGCGAG
Primer 53	AACAGCTCCTCGCCTTGCTCACCGACTTTTGTCCAGGTCCAGG
Primer 54	TGCCGCCAGAACACAGCTGAAGC
Primer 55	GAGTGCATCGGGCCTTGC
Primer 56	CGCAAGGCCGATCGACTCCTGTGTGGCTTCGATCCTACCGCTGAC
Primer 57	ACTATAACCCTGCAGCAGGTTG
Primer 58	ACCTGCTGCAGGTTATAGTGCCGCTGTGCCAACAAACAGTACGGTGTGGTGC
Primer 59	TCCACCACACTGGACTAGTGATCCTTATCATTAAACGGGCCCTTCCAGC

2.2 Working vector sequences. Related to STAR methods.

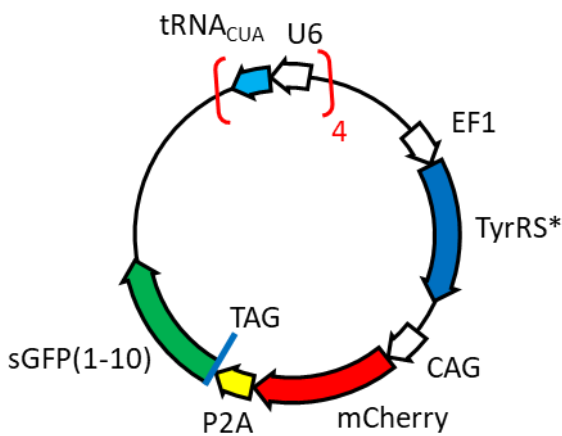
Vector 1 has **kanamycin** resistance; all other vectors have **ampicillin** resistance. Gene sequences are colour-coded using the following: **U6 promoter**, **PyIT** (anticodon **highlighted**), **EF1 promoter**, **PyIRS**, **CAG promoter and chimeric intron**, **eGFP** (150th codon **highlighted**),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

2.2.1 Vector 1: Pyl tRNA^{CUA(U25C)}



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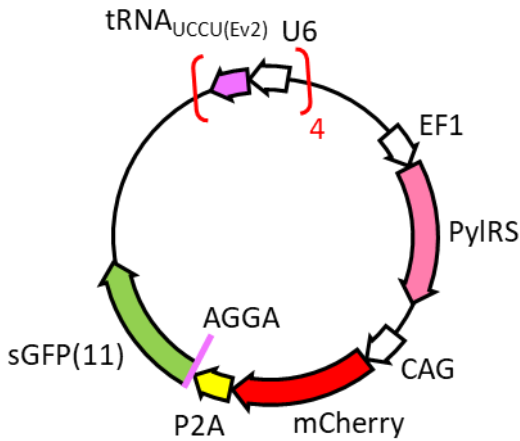
2.2.2 Vector 55: 4x(Tyr tRNA_{CUA}) TyrRS* mCherry-P2A-TAG-sGFP(1-10)



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2.2.3 Vector 56: 4x(Pyl tRNA_{UCCU(Ev2)}) PylRS mCherry-P2A-AGGA-sGFP(11)

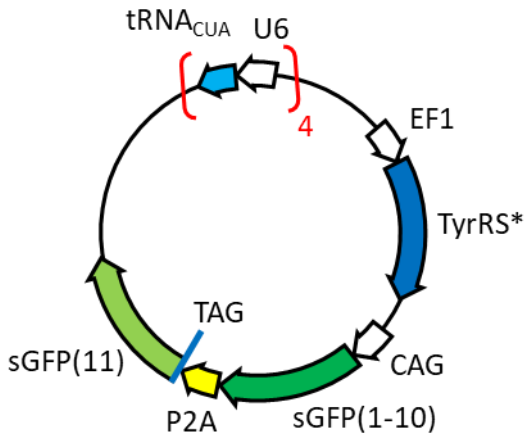


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2.2.4 Vector 57: 4x(Tyr tRNA_{CUA}) TyrRS* sGFP(1-10)-TAG-sGFP(11)

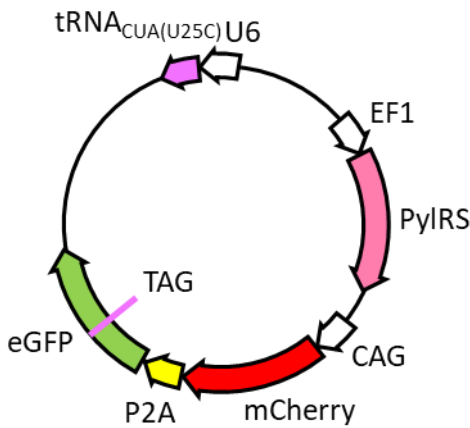


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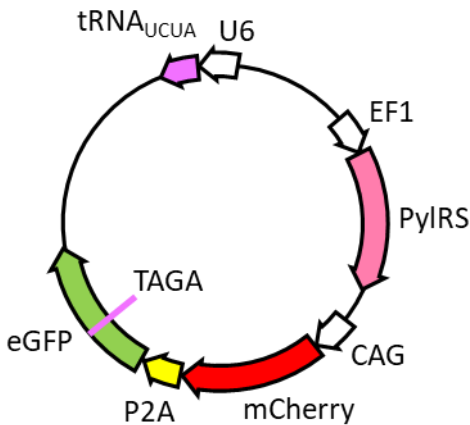
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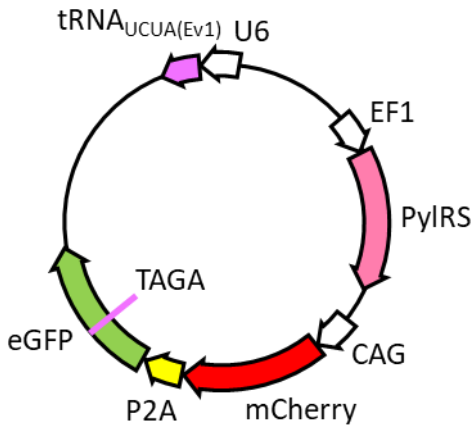
2.2.6 Vector 59: Pyl tRNA_{UCUA} PylRS mCherry-P2A-eGFP(150TAGA)



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2.2.7 Vector 60: Pyl tRNA_{UCUA(Ev1)} PyIRS mCherry-P2A-eGFP(150TAGA)

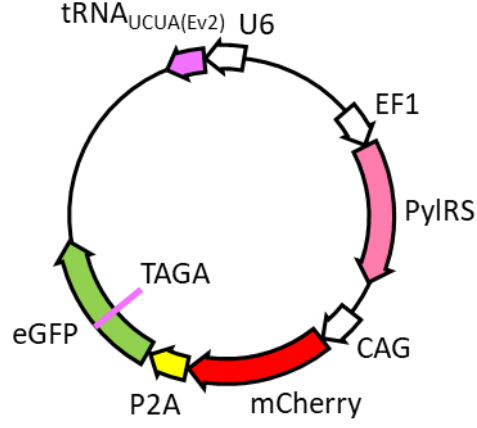


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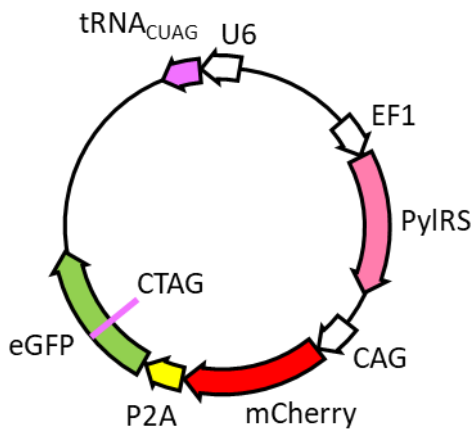
2.2.8 Vector 61: Pyl tRNA_{UCUA(Ev2)} PylRS mCherry-P2A-eGFP(150TAGA)



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

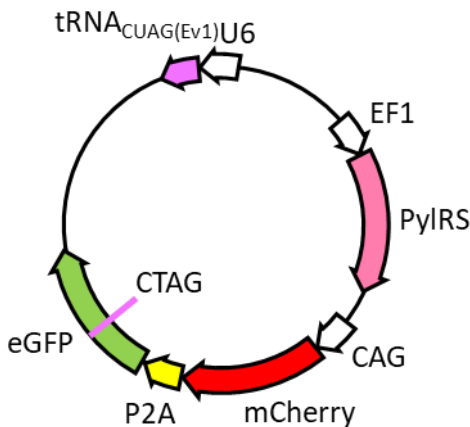


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2.2.10 Vector 63: Pyl tRNA_{CUAG(Ev1)} PylRS mCherry-P2A-eGFP(150CTAG)

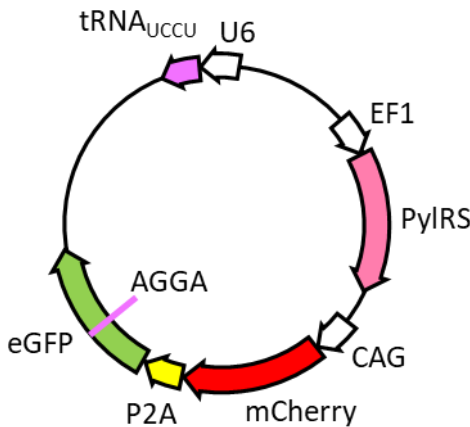


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2.2.11 Vector 64: Pyl tRNA_{UCCU} PylRS mCherry-P2A-eGFP(150AGGA)

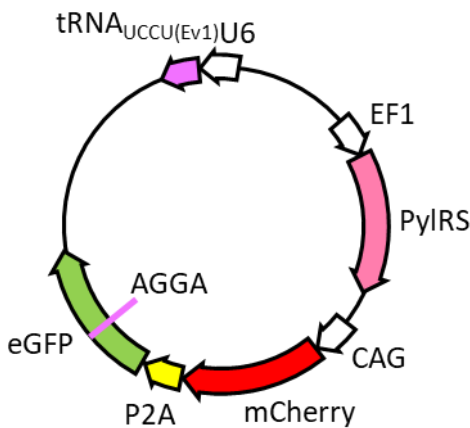


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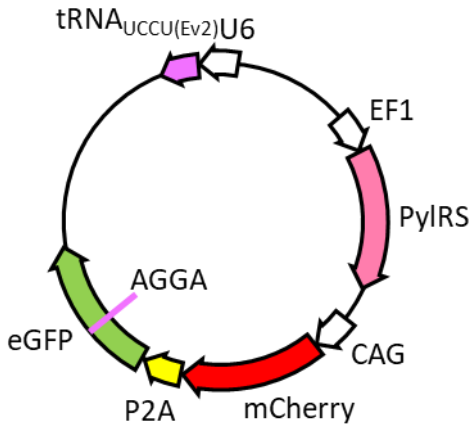
2.2.12 Vector 65: Pyl tRNA_{UCCU(Ev1)} PylRS mCherry-P2A-eGFP(150AGGA)



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

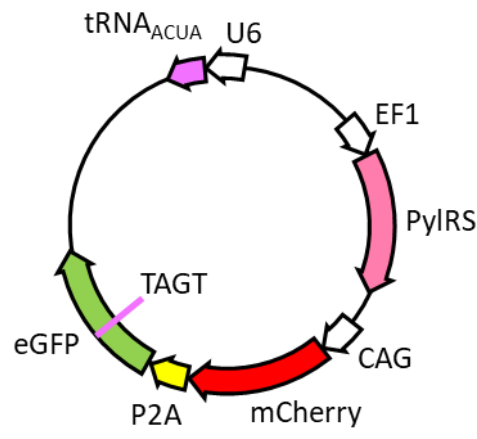


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2.2.14 Vector 67: Pyl tRNA_{ACUA} PylRS mCherry-P2A-eGFP(150TAGT)

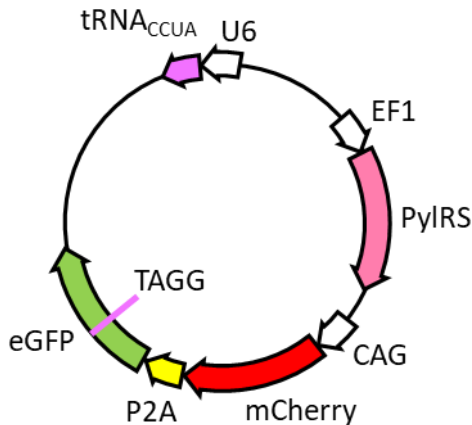


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2.2.15 Vector 68: Pyl tRNA_{CCUA} PylRS mCherry-P2A-eGFP(150TAGG)

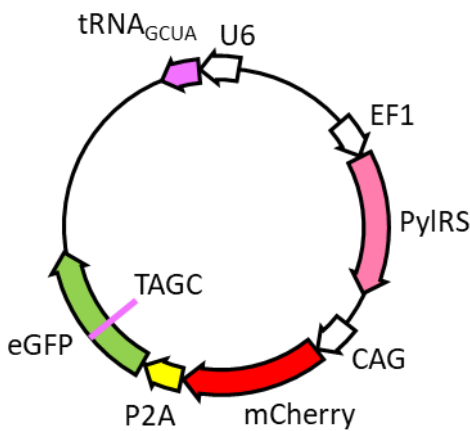


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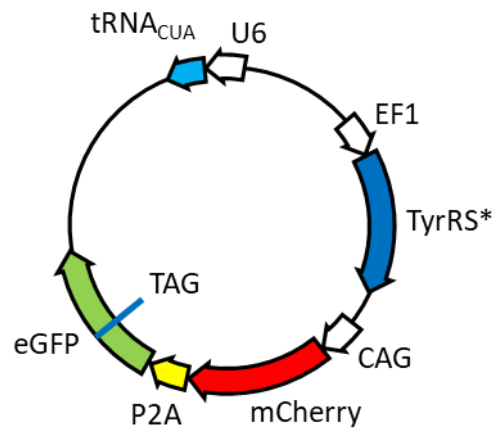
2.2.16 Vector 69: Pyl tRNA_{GCUA} PylRS mCherry-P2A-eGFP(150TAGC)



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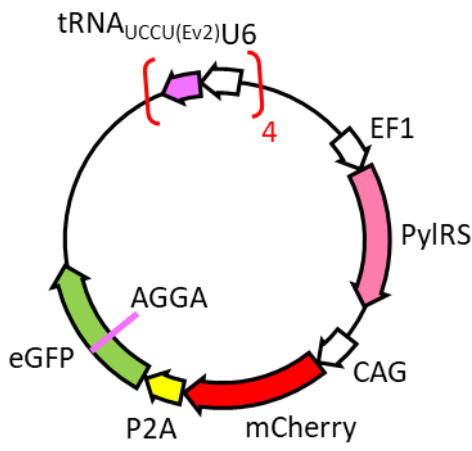
2.2.17 Vector 71: Tyr tRNA_{CUA} TyrRS* mCherry-P2A-eGFP(150TAG)



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2.2.18 Vector 73: 4x(PyI tRNA_{UCCU(Ev2)}) PyIRS mCherry-P2A-eGFP(150AGGA)

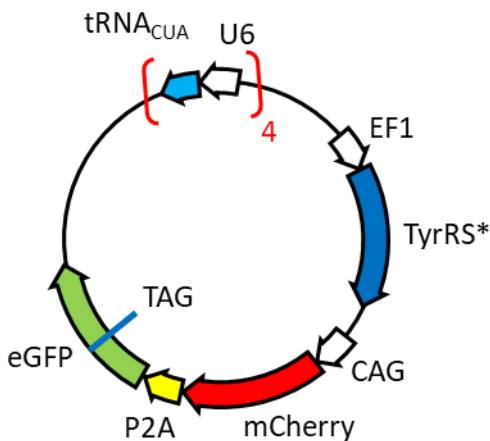


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2.2.19 Vector 74: 4x(Tyr tRNA_{CUA}) TyrRS* mCherry-P2A-eGFP(150TAG)



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