Electronic Supplementary Information

For

# Rational optimization of amber suppressor tRNAs toward efficient incorporation of a non-natural amino acid into protein in a eukaryotic wheat germ extract

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**Sequences of primers and templates in PCRs**

(written from 5’ (left) to 3’ (right); underlined: *Spe* I site; X: 2’-OMe-G.)

Forward primer in the 5’ segment amplification for **amber-mRNA**

CCGGCGCCAGATGGCTAGACA

Reverse primer in the 5’ segment amplification for **amber-mRNA**

GACACTACTAGTGATATCTTGGTGATGTAGA

1st forward primer in the 3’ segment amplification for **amber-mRNA**

AGATACAGCAGCGGCCTGGTGCCGCGCGGCAGCCACGTGAGCAAGGGCGAGGAG

2nd forward primer in the 3’ segment amplification for **amber-mRNA**

GACACTACTAGTATGGCCCATCACCATCACCATCATTAGAGATACAGCAGCGGCCT

1st and 2nd reverse primer in the 3’ segment amplification for **amber-mRNA**

AGCTGTTTGCGCGTCTGAAAG

Forward primer in the final PCR for **amber-mRNA**

CATACGATTTAGGTGACACT

Reverse primer in the final PCR for **amber-mRNA**

TTAGCGGCTTTATTGATTGC

Forward primer for flexizymes

ACGCATATGTAATACGACTCACTATAGGATCGAAAGATTTCCGCA

Reverse primer for a flexizyme with $U_{45}$

TTTTTTTTTTTTTTTTTTTTACCTAACGCCAATACCCTTTCG

Reverse primer for a flexizyme with $G_{45}$

TTTTTTTTTTTTTTTTTTTTCCCTAACGCCAATACCCTTTCG

Template for flexizymes

GGATCGAAAGATTTCCGCAGGCCCGAAAGGGTATTGGCGTTAGGT

Forward primer for **t86**

GAAATTAATACGACTCACTATAGGAGAGATGGCTGAGTG

Reverse primer for **t86**

TXGCGGAGAGAGAGGGATTCGAACCCTCGATAGTTC

Template for **t86**

GGAGAGATGGCTGAGTGGTTGATAGCTGCGGTCTCTAAAACCGCTATAGTTCTAGGAACTA
TCGAGGGTTCGAATCCCT

Forward primer for **tAla1**

GTAATACGACTCACTATAGGGGCTATAGCTCAGCTGG

Reverse primer for **tAla1**

TXGTGGAGCTAAGCGGGATCG

Template for **tAla1**

GGGGCTATAGCTCAGCTGGGAGAGCGCTTGCATCTAATGCAAGAGGTCAGCGGTTCGATCC
CGCTTAGCTCCACCA

Forward primer for **tAla2**

GTAATACGACTCACTATAGGGGCTATAGCTCAGCTGG

Reverse primer for **tAla2**

TXGTGGAGCTATGCGGGATCG

Template for **tAla2**

GGGGCTATAGCTCAGCTGGGAGAGCGCCTGCTTCTAACGCAGGAGGTCTGCGGTTCGATCC
CGCATAGCTCCACCA

Forward primer for **tArg1**

GTAATACGACTCACTATAGCATCCGTAGCTCAGCTGG

Reverse primer for **tArg1**

TXGTGCATCCGGGAGGATTC

Template for **tArg1**

GCATCCGTAGCTCAGCTGGATAGAGTACTCGGCTCTAAACCGAGCGGTCGGAGGTTCGAAT
CCTCCCGGATGCACCA

Forward primer for **tArg2**

GTAATACGACTCACTATAGCGCCCGTAGCTCAGCTGG

Reverse primer for **tArg2**

TXGCGCGCCCGACAGGATTC

Template for **tArg2**

GCGCCCGTAGCTCAGCTGGATAGAGCGCTGCCCTCTAGAGGCAGAGGTCTCAGGTTCGAAT
CCTGTCGGGCGCGCCA

Forward primer for **tArg3**

GTAATACGACTCACTATAGTCCTCTTAGTTAAATGG

Reverse primer for **tArg3**

TXGTGTCCCCTGCAGGAATC

Template for **tArg3**

GTCCTCTTAGTTAAATGGATATAACGAGCCCCTCTAAAGGGCTAATTGCAGGTTCGATTCC
TGCAGGGGACACCA

Forward primer for **tArg4**

GTAATACGACTCACTATAGCGCCCTTAGCTCAGTTG

Reverse primer for **tArg4**

TXGCGCGCCCTGCAGGATTC

Template for **tArg4**

GCGCCCTTAGCTCAGTTGGATAGAGCAACGACCTCTAAAGTCGTGGGCCGCAGGTTCGAAT
CCTGCAGGGCGCGCCA

Forward primer for **tAsp**

GTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGT

Reverse primer for **tAsp**

TXGCGGAACGGACGGGACTC

Template for **tAsp**

GGAGCGGTAGTTCAGTCGGTTAGAATACCTGCCTCTAACGCAGGGGGTCGCGGGTTCGAGT
CCCGTCCGTTCCGCCA

Forward primer for **tCys**

GTAATACGACTCACTATAGGCGCGTTAACAAAGCGG

Reverse primer for **tCys**

TXGAGGCGCGTTCCGGAGTC

Template for **tCys**

GGCGCGTTAACAAAGCGGTTATGTAGCGGATTCTAAATCCGTCTAGTCCGGTTCGACTCCG
GAACGCGCCTCCA

Forward primer for **tGlu**

GTAATACGACTCACTATAGTCCCCTTCGTCTAGAGG

Reverse primer for **tGlu**

TXGCGTCCCCTAGGGGATTC

Template for **tGlu**

GTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTCTAACGGCGGTAACAGGGGTTCGAATC
CCCTAGGGGACGCCA

Forward primer for **tGly1**

GTAATACGACTCACTATAGCGGGAATAGCTCAGTTG

Reverse primer for **tGly1**

TXGAGCGGGAAACGAGACTC

Template for **tGly1**

GCGGGAATAGCTCAGTTGGTAGAGCACGACCTTCTAAAGGTCGGGGTCGCGAGTTCGAGT
CTCGTTTCCCGCTCCA

Forward primer for **tGly2**

GTAATACGACTCACTATAGCGGGCGTAGTTCAATGG

Reverse primer for **tGly2**

TXGAGCGGGCGAAGGGAATC

Template for **tGly2**

GCGGGCGTAGTTCAATGGTAGAACGAGAGCTTCTAAAGCTCTATACGAGGGTTCGATTCCC
TTCGCCCGCTCCA

Forward primer for **tGly3**

GTAATACGACTCACTATAGCGGGCATCGTATAATGG

Reverse primer for **tGly3**

TXGAGCGGGCAGCGGGAATC

Template for **tGly3**

GCGGGCATCGTATAATGGCTATTACCTCAGCCTCTAAAGCTGATGATGCGGGTTCGATTCC
CGCTGCCCGCTCCA

Forward primer for **tHis**

GTAATACGACTCACTATAGTGGCTATAGCTCAGTTG

Reverse primer for **tHis**

TXGGGTGGCTAATGGGATTC

Template for **tHis**

GTGGCTATAGCTCAGTTGGTAGAGCCCTGGATTCTAATTCCAGTTGTCGTGGGTTCGAATCC
CATTAGCCACCCCA

Forward primer for **tLeu1**

GTAATACGACTCACTATAGCCGAGGTGGTGGAATTG

Reverse primer for **tLeu1**

TXGTACCGAGGACGGGACTTGAACCCGTAAG

Template for **tLeu1**

GCCGAGGTGGTGGAATTGGTAGACACGCTACCTTCTAGTGGTAGTGCCCAATAGGGCTTAC
GGGTTCAAGTCCCGTC

Forward primer for **tLeu2**

GTAATACGACTCACTATAGCGAAGGTGGCGGAATTG

Reverse primer for **tLeu2**

TXGTGCGAGGGGGGGGACTTGAACCCCCACGTC

Template for **tLeu2**

GCGAAGGTGGCGGAATTGGTAGACGCGCTAGCTTCTAGTGTTAGTGTCCTTACGGACGTGG
GGGTTCAAGTCCC

Forward primer for **tLeu3**

GTAATACGACTCACTATAGCGGGAGTGGCGAAATTG

Reverse primer for **tLeu3**

TXGTGCGGGAGGCGAGACTTGAACTCGCACAC

Template for **tLeu3**

GCGGGAGTGGCGAAATTGGTAGACGCACCAGATTCTAGTTCTGGCGCCGCAAGGTGTGCG
AGTTCAAGTCTCGC

Forward primer for **tLeu4**

GTAATACGACTCACTATAGCCGAAGTGGCGAAATCG

Reverse primer for **tLeu4**

TXGTGCCGAAGGCCGGACTCGAACCGGCACGT

Template for **tLeu4**

GCCGAAGTGGCGAAATCGGTAGACGCAGTTGATTCTAAATCAACCGTAGAAATACGTGCC
GGTTCGAGTCCGGCCT

Forward primer for **tLeu5**

GTAATACGACTCACTATAGCCCGGATGGTGGAATCGGT

Reverse primer for **tLeu5**

TXGTACCCGGAGCGGGACTTGAACCCGCACA

Template for **tLeu5**

GCCCGGATGGTGGAATCGGTAGACACAAGGGATTCTAAATCCCTCGGCGTTCGCGCTGTGC
GGGTTCAAGTCCCGCT

Forward primer for **tLys**

GTAATACGACTCACTATAGGGTCGTTAGCTCAGTTGG

Reverse primer for **tLys**

TXGTGGGTCGTGCAGGATTC

Template for **tLys**

GGGTCGTTAGCTCAGTTGGTAGAGCAGTTGACTCTAAATCAATTGGTCGCAGGTTCGAATC
CTGCACGACCCACCA

Forward primer for **tMet1**

GTAATACGACTCACTATAGGCTACGTAGCTCAGTTG

Reverse primer for **tMet1**

TXGTGGCTACGACGGGATTC

Template for **tMet1**

GGCTACGTAGCTCAGTTGGTTAGAGCACATCACTCTAAATGATGGGGTCACAGGTTCGAAT
CCCGTCGTAGCCACCA

Forward primer for **tMet2**

GTAATACGACTCACTATAGGCCCTTTAGCTCAGTGG

Reverse primer for **tMet2**

TXGTGGCCCTTGCTGGACTTG

Template for **tMet2**

GGCCCTTTAGCTCAGTGGTTAGAGCAGGCGACTCTAAATCGCTTGGTCGCTGGTTCAAGTC
CAGCAAGGGCCACCA

Forward primer for **tMet3**

GTAATACGACTCACTATAGGCCCCTTAGCTCAGTGG

Reverse primer for **tMet3**

TXGTGGCCCCTGCTGGACTTG

Template for **tMet3**

GGCCCCTTAGCTCAGTGGTTAGAGCAGGCGACTCTAAATCGCTTGGTCGCTGGTTCAAGTC
CAGCAGGGGCCACCA

Forward primer for **tPhe**

GTAATACGACTCACTATAGCCCGGATAGCTCAGTCGGT

Reverse primer for **tPhe**

TXGTGCCCGGACTCGGAATC

Template for **tPhe**

GCCCGGATAGCTCAGTCGGTAGAGCAGGGGATTCTAAATCCCCGTGTCCTTGGTTCGATTC
CGAGTCCGGGCACCA

Forward primer for **tSec**

GTAATACGACTCACTATAGAAGATCGTCGTCTCCGGT

Reverse primer for **tSec**

TXGGGAAGATCACAGGAGTCGAACCTGCCCGGGACCGCT

Template for **tSec**

GAAGATCGTCGTCTCCGGTGAGGCGGCTGGACTCTAAATCCAGTTGGGGCCGCCAGCGGTC
CCGGGCAGGTTCGA

Forward primer for **tSer1**

GAAATTAATACGACTCACTATAGGAGAGATGCCGGAGCGGCTGAAC

Reverse primer for **tSer1**

TXGCGGAGAGAGGGGGATTTG

Template for **tSer1**

GGAGAGATGCCGGAGCGGCTGAACGGACCGGTCTCTAAAACCGGAGTAGGGGCAACTCTA
CCGGGGGTTCAAATCCCCCTCTCTCCGCCA

Forward primer for **tSer2**

GTAATACGACTCACTATAGGTGAGGTGTCCGAGTGG

Reverse primer for **tSer2**

TXGCGGTGAGGGGGGGATTCGAACCCCCGATAC

Template for **tSer2**

GGTGAGGTGTCCGAGTGGCTGAAGGAGCACGCCTCTAAAGTGTGTATACGGCAACGTATC
GGGGGTTCGAATCC

Forward primer for **tSer3**

GTAATACGACTCACTATAGGAAGTGTGGCCGAGCGGT

Reverse primer for **tSer3**

TXGCGGAAGCGCAGAGATTCGAACTCTGGAAC

Template for **tSer3**

GGAAGTGTGGCCGAGCGGTTGAAGGCACCGGTCTCTAAAACCGGCGACCCGAAAGGGTTC
CAGAGTTCGAATCTC

Forward primer for **tSer4**

GTAATACGACTCACTATAGGTGAGGTGGCCGAGAG

Reverse primer for **tSer4**

TXGCGGTGAGGCGGGGATTCGAACCCCGGATGCAG

Template for **tSer4**

GGTGAGGTGGCCGAGAGGCTGAAGGCGCTCCCCTCTAAAGGGAGTATGCGGTCAAAAGCT
GCATCCGGGGTTCGAAT

Forward primer for **tThr1**

GTAATACGACTCACTATAGCTGATATAGCTCAGTTG

Reverse primer for **tThr1**

TXGTGCTGATAGGCAGATTC

Template for **tThr1**

GCTGATATAGCTCAGTTGGTAGAGCGCACCCTTCTAAAGGGTGAGGTCGGCAGTTCGAATC
TGCCTATCAGCACCA

Forward primer for **tThr2**

GTAATACGACTCACTATAGCTGATATGGCTCAGTTG

Reverse primer for **tThr2**

TXGTGCTGATACCCAGAGTC

Template for **tThr2**

GCTGATATGGCTCAGTTGGTAGAGCGCACCCTTCTAAAGGGTGAGGTCCCCAGTTCGACTC
TGGGTATCAGCACCA

Forward primer for **tThr3**

GTAATACGACTCACTATAGCCGATATAGCTCAGTTG

Reverse primer for **tThr3**

TXGTGCCGATAATAGGAGTC

Template for **tThr3**

GCCGATATAGCTCAGTTGGTAGAGCAGCGCATTCTAAATGCGAAGGTCGTAGGTTCGACTC
CTATTATCGGCACCA

Forward primer for **tThr4**

GTAATACGACTCACTATAGCTCAAGTAGTTAAAAATG

Reverse primer for **tThr4**

TXGTGCCGATAATAGGAGTC

Template for **tThr4**

GCTCAAGTAGTTAAAAATGCATTAACATCGCATTCTAAATGCGAAGGTCGTAGGTTCGACT
CCTATTATCGGCACCA

Forward primer for **tThr5**

GTAATACGACTCACTATAGCCGACTTAGCTCAGTAG

Reverse primer for **tThr5**

TXGTGCCGACTACCGGAATC

Template for **tThr5**

GCCGACTTAGCTCAGTAGGTAGAGCAACTGACTCTAAATCAGTAGGTCACCAGTTCGATTC
CGGTAGTCGGCACCA

Forward primer for **tTyr1**

GTAATACGACTCACTATAGGTGGGGTTCCCGAGC

Reverse primer for **tTyr1**

TXGTGGTGGGGGAAGGATTCGAACCTTCGAAGTCT

Template for **tTyr1**

GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCACAGACTTCGAA
GGTTCGAATCCTTCC

Forward primer for **tTyr2**

GTAATACGACTCACTATAGGTGGGGTTCCCGAGC

Reverse primer for **tTyr2**

TXGTGGTGGGGGAAGGATTCGAACCTTCGAAGTCG

Template for **tTyr2**

GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAA
GGTTCGAATCCTTCC

Forward primer for **tVal1**

GTAATACGACTCACTATAGCGTCCGTAGCTCAGTTG

Reverse primer for **tVal1**

TXGTGCGTCCGAGTGGACT

Template for **tVal1**

GCGTCCGTAGCTCAGTTGGTTAGAGCACCACCTTCTAATGGTGGGGGTCGGTGGTTCGAGT
CCACTCGGACGCACCA

Forward primer for **tVal2**

GTAATACGACTCACTATAGCGTTCATAGCTCAGTTG

Reverse primer for **tVal2**

TXGTGCGTTCAATTGGACTC

Template for **tVal2**

GCGTTCATAGCTCAGTTGGTTAGAGCACCACCTTCTAATGGTGGGGGTCGTTGGTTCGAGT
CCAATTGAACGCACCA

Forward primer for **tVal3**

GTAATACGACTCACTATAGGGTGATTAGCTCAGCTGG

Reverse primer for **tVal3**

TXGTGGGTGATGACGGGAT

Template for **tVal3**

GGGTGATTAGCTCAGCTGGGAGAGCACCTCCCTCTAAAGGAGGGGGTCGGCGGTTCGATC
CCGTCATCACCCACCA

Forward primer for **mtAsn**

ACGCATATGTAATACGACTCACTATAGCCTCTGTAGTTCAGTCGGT

Reverse primer for **mtAsn**

TXGTGCCTCTGACTGGACTC

Template for **mtAsn**

GCCTCTGTAGTTCAGTCGGTAGAACGGCGGACTCTAAATCCGTATGTCACTGGTTCGAGTC
CAGTCAGAGGCACCA

Forward primer for **tGly3-$U_{31}A_{39}$**

GTAATACGACTCACTATAGCGGGCATCGTATAATGG

Reverse primer for **tGly3-$U_{31}A_{39}$**

TXGAGCGGGCAGCGGGAATC

Template for **tGly3-$U_{31}A_{39}$**

GCGGGCATCGTATAATGGCTATTACCTCAGTCTCTAAAACTGATGATGCGGGTTCGATTCC
CGCTGCCCGCTCCA

Forward primer for **tGly3-$U_{31}A_{39}$-$A_{51}U_{63}$**

GTAATACGACTCACTATAGCGGGCATCGTATAATGG

Reverse primer for **tGly3-$U_{31}A_{39}$-$A_{51}U_{63}$**

TXGAGCGGGCAGCAGGAATC

Template for **tGly3-$U_{31}A_{39}$-$A_{51}U_{63}$**

GCGGGCATCGTATAATGGCTATTACCTCAGTCTCTAAAACTGATGATGCAGGTTCGATTCC
TGCTGCCCGCTCCA

Forward primer for **tLys-$U_{31}A_{39}$**

GTAATACGACTCACTATAGGGTCGTTAGCTCAGTTGG

Reverse primer for **tLys-$U_{31}A_{39}$**

TXGTGGGTCGTGCAGGATTC

Template for **tLys-$U_{31}A_{39}$**

GGGTCGTTAGCTCAGTTGGTAGAGCAGTTGTCTCTAAAACAATTGGTCGCAGGTTCGAATC
CTGCACGACCCACCA

Forward primer for **tLys-$U_{31}A_{39}$-$G_{51}C_{63}$**

GTAATACGACTCACTATAGGGTCGTTAGCTCAGTTGG

Reverse primer for **tLys-$U_{31}A_{39}$-$G_{51}C_{63}$**

TXGTGGGTCGTGCGGGATTCGAAC

Template for **tLys-$U_{31}A_{39}$-$G_{51}C_{63}$**

GGGTCGTTAGCTCAGTTGGTAGAGCAGTTGTCTCTAAAACAATTGGTCGCGGGTTCGAATC
CCGCACGACCCACCA

Forward primer for **tLys-opt**

GTAATACGACTCACTATAGGGTCCATAGCTCAGTTGGT

Reverse primer for **tLys-opt**

TXGTGGGTCCAGCGGGATTC

Template for **tLys-opt**

GGGTCCATAGCTCAGTTGGTAGAGCAGTTGTCTCTAAAACAATTGGTCGCGGGTTCGAATC
CCGCTGGACCCACCA

**Whole sequence of pHis-TAG-RY-YPet**

(shaded: SP6 promoter; underlined: *Spe* I site; purple: start codon; red: amber codon; green: in-frame original start codon; orange: YPet gene; blue: stop codon in the presence of an efficient amber sup-tRNA)

CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCAT

TTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGAT

AGGGTTGAGTGGCCGCTACAGGGCGCTCCCATTCGCCATTCAGGCTGCGCAACTGTTGGGA

AGGGCGTTTCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGC

AAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCC

AGTGAGCGCGACGTAATACGACTCACTATAGGGCGAATTGAAGGAAGGCCGTCAAGGCCG

CATGGTACCATACGATTTAGGTGACACTATAGAACTCACCTATCTCCCCAACACCTAATAA

CATTCAATCACTCTTTCCACTAACCACCTATCTACATCACCAAGATATCACTAGTATGGCCC

ATCACCATCACCATCATTAGAGATACAGCAGCGGCCTGGTGCCGCGCGGCAGCCACATGGT

GAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGA

CGTGAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAA

GCTGACCCTGAAGCTGCTGTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG

ACCACCCTGGGCTACGGCGTGCAGTGCTTCGCCCGCTACCCCGACCACATGAAGCAGCACG

ACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGA

CGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCG

CATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGA

GTACAACTACAACAGCCACAACGTCTATATCACCGCCGACAAGCAGAAGAACGGCATCAA

GGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCGGCGTGCAGCTCGCCGACCACTA

CCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGC

TACCAGTCCGCCCTGTTCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGT

TCCTGACCGCCGCCGGGATCACTGAGGGCATGAACGAGCTGTACAAGTAACTCGAGCTCCT

GGGCCTCATGGGCCTTCCTTTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTG

CATTAACATGGTCATAGCTGTTTCCTTGCGTATTGGGCGCTCTCCGCTTCCTCGCTCACTGA

CTCGCTGCGCTCGGTCGTTCGGGTAAAGCCTGGGGTGCCTAATGAGCAAAAGGCCAGCAA

AAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTG

ACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAA

GATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTT

ACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTG

TAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCC

GTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGAC

ACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAG

GCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATT

TGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCC

GGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCA

GAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAA

CGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATC

CTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTG

ACAGTTATTAGAAAAATTCATCCAGCAGACGATAAAACGCAATACGCTGGCTATCCGGTGC

CGCAATGCCATACAGCACCAGAAAACGATCCGCCCATTCGCCGCCCAGTTCTTCCGCAATA

TCACGGGTGGCCAGCGCAATATCCTGATAACGATCCGCCACGCCCAGACGGCCGCAATCA

ATAAAGCCGCTAAAACGGCCATTTTCCACCATAATGTTCGGCAGGCACGCATCACCATGGG

TCACCACCAGATCTTCGCCATCCGGCATGCTCGCTTTCAGACGCGCAAACAGCTCTGCCGG

TGCCAGGCCCTGATGTTCTTCATCCAGATCATCCTGATCCACCAGGCCCGCTTCCATACGGG

TACGCGCACGTTCAATACGATGTTTCGCCTGATGATCAAACGGACAGGTCGCCGGGTCCAG

GGTATGCAGACGACGCATGGCATCCGCCATAATGCTCACTTTTTCTGCCGGCGCCAGATGG

CTAGACAGCAGATCCTGACCCGGCACTTCGCCCAGCAGCAGCCAATCACGGCCCGCTTCGG

TCACCACATCCAGCACCGCCGCACACGGAACACCGGTGGTGGCCAGCCAGCTCAGACGCG

CCGCTTCATCCTGCAGCTCGTTCAGCGCACCGCTCAGATCGGTTTTCACAAACAGCACCGG

ACGACCCTGCGCGCTCAGACGAAACACCGCCGCATCAGAGCAGCCAATGGTCTGCTGCGC

CCAATCATAGCCAAACAGACGTTCCACCCACGCTGCCGGGCTACCCGCATGCAGGCCATCC

TGTTCAATCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATG

AGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTC

CCCGAAAAGTGCCAC

**Optimal conditions for efficient AcPhe incorporation with tLys-opt in WGE**

We searched for the optimal conditions for AcPhe incorporation with **tLys-opt** (**Fig. S2A**) to obtain a larger amount of AcPhe-incorporated protein in WGE. Because **tLys-opt** that has released AcPhe is not reused, the amount of AcPhe-charged **tLys-opt** should have a larger effect on the efficiency of AcPhe incorporation. We thus investigated the dependence of suppression efficiency on **tLys-opt** concentration (**Fig. S2B**). The results showed that the suppression efficiency significantly increased in proportion to the concentration of **tLys-opt** (+AcPhe charging) up to 4 μM, whereas it plateaued at approx. 1 μM in the case of reusable **t86**.[1] Consequently, the maximum suppression efficiency was twice that of **t86** (approx. 100% of the translation efficiency of **amber-free mRNA**, in which the amber codon in **amber-mRNA** was eliminated[2]).

To further improve the suppression efficiency, we added an eRF1-binding aptamer (**apt12**),[2,3] which facilitates sup-tRNAs to suppress the nonsense codons by blocking eRF1, to the translation solution including the optimal amount of **tLys-opt** with AcPhe charging. However, the suppression efficiency gradually decreased in response to the aptamer concentration (**Fig. S2C**). This is probably because the amount of sup-tRNA was just enough to completely win the competition with eRF1 for at least a 1-h translation, as indicated by the high translation efficiency, which was comparable to that of **amber-free mRNA**, and also because the aptamer also inhibited translation termination at the terminal codon (UAA) of the YPet gene.[2]

Moreover, on the basis of a report that polyguanylic acid, poly(G), enhanced translation efficiency by inhibiting RNases in WGE,[4] we examined the effect of poly(G) on amber suppression in WGE. The results showed the suppression efficiency was enhanced 1.8-fold at a maximum with 100 ng/μL of poly(G) (**Fig. S2D**). This translational enhancement by poly(G) was larger than that in **amber-free mRNA** (1.4-fold at a maximum), probably because the degradation of not only mRNA but also AcPhe-charged **tLys-opt** was inhibited by poly(G).[1]

## Supplementary Figures

| isotype | anticodon | $N_{73}$ | gene copy | sup-tRNA |
|---|---|---|---|---|
| Ala | GGC | A | 2 | **tAla1** |
| | UGC | A | 3 | **tAla2** |
| Arg | ACG | A | 4 | **tArg1** |
| | CCG | G | 1 | **tArg2** |
| | CCU | A | 1 | **tArg3** |
| | UCU | G | 1 | **tArg4** |
| Asp | GUC | G | 3 | **tAsp** |
| Cys | GCA | U | 1 | **tCys** |
| Glu | UUC | G | 4 | **tGlu** |
| Gly | GCC | U | 4 | **tGly1** |
| | CCC | U | 1 | **tGly2** |
| | UCC | U | 1 | **tGly3** |
| His | GUG | C | 1 | **tHis** |
| Leu | GAG | A | 1 | **tLeu1** |
| | CAG | A | 4 | **tLeu2** |
| | UAG | A | 1 | **tLeu3** |
| | CAA | A | 1 | **tLeu4** |
| | UAA | A | 1 | **tLeu5** |
| Lys | UUU | A | 6 | **tLys** |

| isotype | anticodon | $N_{73}$ | gene copy | sup-tRNA |
|---|---|---|---|---|
| Met | CAU(3) | A | 2 | **tMet1** |
| | | A | 1 | **tMet2** |
| | | A | 1 | **tMet3** |
| Phe | GAA | A | 2 | **tPhe** |
| Sec | UCA | C | 1 | **tSec** |
| Ser | CGA | G | 1 | **tSer1** |
| | GGA | G | 2 | **tSer2** |
| | UGA | G | 1 | **tSer3** |
| | GCU | G | 1 | **tSer4** |
| Thr | GGU(2) | A | 1 | **tThr1** |
| | | A | 1 | **tThr2** |
| | CGU(2) | A | 1 | **tThr3** |
| | | A | 1 | **tThr4** |
| | UGU | A | 1 | **tThr5** |
| Tyr | GUA(2) | A | 1 | **tTyr1** |
| | | A | 2 | **tTyr2** |
| Val | GAC(2) | A | 1 | **tVal1** |
| | | A | 1 | **tVal2** |
| | UAC | A | 5 | **tVal3** |

**Figure S1.** Classification of *E. coli* K-12 tRNAs with a 5' terminal $G_1$ by their sequences, which are described in the genomic tRNA database (http://gtrnadb.ucsc.edu/). The numbers in parentheses in the 'anticodon' column indicate different types of tRNAs with the same anticodon. $N_{73}$ represents the discriminator base. The anticodons of all these 38 types of *E. coli* tRNAs were converted to CUA for the amber codon to prepare amber sup-tRNA candidates ('sup-tRNA' column).

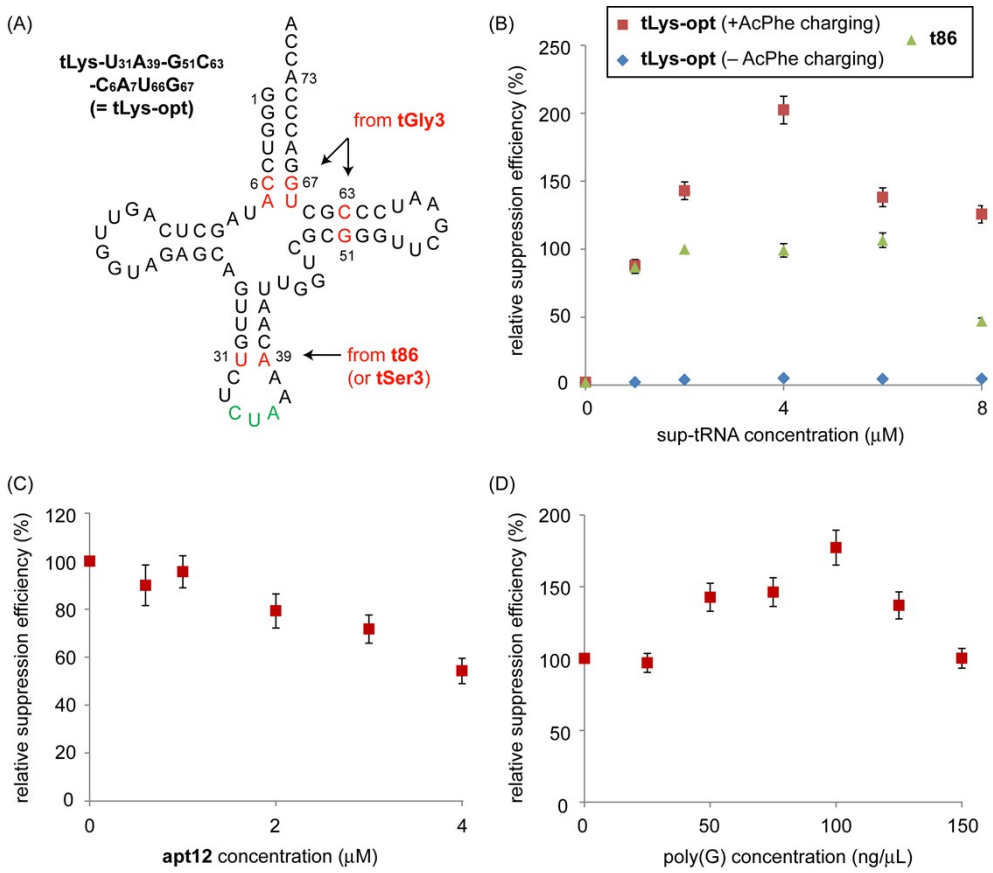


**Figure S2.** Optimization of translation conditions for AcPhe incorporation with **tLys-opt**. (A) The secondary structure of **tLys-opt**. The green and red characters indicate the anticodon for the amber codon and bases different from those in the parent **tLys**, respectively. (B) The relative suppression efficiency in the presence of various concentrations of **tLys-opt** (with or without AcPhe charging) or **t86**, compared to that with 2 μM **t86**. (C and D) The relative suppression efficiency in the presence of 4 μM **tLys-opt** with AcPhe charging and various concentrations of **apt12** (C) or poly(G) (D).

(A)

| sup-tRNA | $N_{31}$-$N_{39}$ (in the anticodon stem) | $N_{49}NNNN_{53}$-$N_{61}NNNN_{65}$ (in the T-stem) | $N_6N_7$-$N_{66}N_{67}$ (in the acceptor stem) |
|---|---|---|---|
| **tSer3** | U-A | CAGAG-CUCUG | UG-CG |
| **t86** | U-A | GAGGG-CCCUC | GA-UC |
| *tLys backbone* | | | |
| **tLys** | A-U | GCAGG-CCUGC | GU-AC |
| **tLys-$U_{31}A_{39}$-tSer3(T)** | U-A | CAGAG-CUCUG | GU-AC |
| **tLys-$U_{31}A_{39}$-tSer3(T+Ac)** | U-A | CAGAG-CUCUG | UG-CG |
| **tLys-$U_{31}A_{39}$-t86(T)** | U-A | GAGGG-CCCUC | GU-AC |
| **tLys-$U_{31}A_{39}$-t86(T+Ac)** | U-A | GAGGG-CCCUC | GA-UC |
| **tLys-opt** | U-A | GCGGG-CCCGC | CA-UG |

(B)

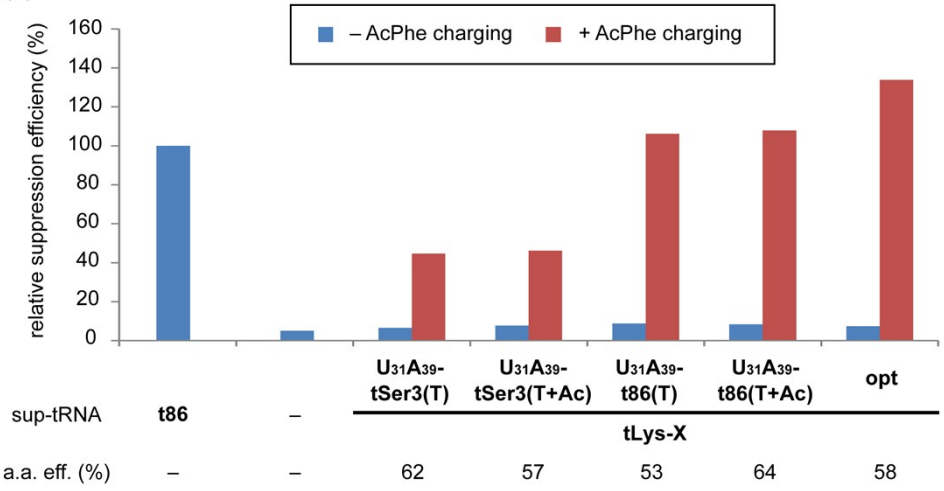


**Figure S3.** (A) The partial sequences of some efficient sup-tRNAs including ones for natural amino acids (**tSer3**, **t86**, **tLys**, **tLys-opt**) and sup-tRNA chimeras between **tLys-$U_{31}A_{39}$** and **tSer3** or **t86**. Red characters in **tLys** variants indicate bases different from those in **tLys**. (B) The relative suppression efficiency of sup-tRNA chimeras in (A) without or with AcPhe charging. The aminoacylation efficiencies (a.a. eff.) are shown below the graph.

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