Supporting Information For:

Modification of Orthogonal tRNAs: Unexpected Consequences for Sense Codon Reassignment

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S.1. Cell Strains

NEB 5-alpha (New England Biolabs): *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*

SB3930 (Yale CGSC): *λ⁻, ΔhisB463*

SS320 (MC1061 F', Lucigen): [F'*proAB+lacIqlacZ*ΔM15 *Tn10* (tetr)] *hsdR mcrB araD139* Δ(*araABC-leu*)7679 *lac*X74 *galUgalK rpsL thi*

CJ236 (New England Biolabs): FΔ*(HindIII)::cat* (Tra^+ Pil^+ Cam^R)/ *ung-1 relA1 dut-1 thi-1 spoT1 mcrA*

S.2 General Materials and Reagents

All restriction enzymes, DNA polymerases, and T4 kinase were purchased from New England Biolabs and used according to the manufacturer's instructions. ATP was purchased from Fisher (BP413-25) and dNTPs were purchased form New England Biolabs (N0447S). DNA isolation was performed using a Thermo Scientific GeneJET plasmid miniprep kit (K0503) according to the manufacturer's protocols. Purification of PCR and mutagenesis reactions were performed using either a Thermo Scientific GeneJET PCR purification kit (K0702) according to the manufacturer's instructions.

LB liquid media (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl) and LB agar plates with 15 g/L agar (TEKNova, A7777) were used unless otherwise noted. Isopropyl-beta-D-thiogalactoside (IPTG) was purchased from Gold Bio (I2481C5). Spectinomycin (Enzo Life Science, BML-A281) was used at 50 μg/mL to maintain the pUltra-based vectors harboring the tRNA and aaRS genes. Carbenicillin (PlantMedia, 40310000-2) was used at 50 μg/mL to maintain the vectors harboring the GFP reporter gene. All bacterial cultures were grown at 37 °C unless otherwise noted.

Electrocompetent stocks of all strains were prepared in-house according to the method of Sambrook and Russell (Sambrook, J., and Russell, D. W. (2001) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory press). Typical transformation efficiencies for electrocompetent cells produced in this way are 10^9 cfu/μg of supercoiled DNA. All electroporation transformations were recovered in SOC (20 g/L tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) for 1 hour at 37 °C with shaking prior to transfer to media containing appropriate antibiotics.

All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, Iowa, USA). All DNA sequencing was performed by Genewiz (Plainfield, NJ, USA).

S.3. DNA Mutagenesis Protocols

QuikChange type Site-Directed Mutagenesis

Non-library site directed mutagenesis for routine cloning and mutation of tRNA anticodons and GFP fluorophore codons was accomplished using a method adapted from the Stratagene QuikChange Multi-Site Directed Mutagenesis protocol. For each 25 μl reaction, 125 ng of mutagenic oligonucleotide was combined with 100 ng of plasmid DNA isolated from NEB 5-alpha cells in 1x Q5 High Fidelity Polymerase with 1x High GC Buffer, 200 μM dNTPs, and 1 unit of Q5 High-Fidelity Polymerase. Reactions were cycled in a PeqSTAR thermocycler (peqLab) for 18 cycles: 98 °C for 20 seconds, and 72 °C for 15 seconds/kb of template plasmid. A final extension at 72 °C was carried out for 5 minutes. 20 units of DpnI enzyme was added directly to the PCR reaction and incubated at 37 °C for 2 hours. Reactions were transformed into electrocompetent NEB 5-alpha without cleanup.

Isolation of single stranded deoxy-uridine containing DNA

DNA libraries were produced with Kunkel mutagenesis using a method adapted from Sidhu and Weiss (Sidhu, S., and Weiss, G. (2004) *Phage display: a practical approach* (Clackson, T. and Lowman, H.B., Ed.). Oxford University Press, USA). Briefly, cultures of CJ236 cells harboring the phagemid to be mutated were grown to an OD_{600} of 0.5 and infected with M13K07 helper phage at a multiplicity of infection of 10:1. The infected culture was transferred into 50-100 mL of LB media with 5 μg/mL chloramphenicol, appropriate antibiotic to maintain the phagemid, and 0.25 μg/mL uridine. Cultures were grown overnight at 30 °C or 37 °C. Cells were pelleted at 17,000 x*g* at 4 °C for 12 minutes in a Sorvall RC 6+ with a Thermo FIBERLite F13-14x50cy rotor. Phage particles were isolated by decanting the supernatant from the pelleted cells into $1/5^{th}$ volume of 20% 8,000 molecular weight polyethylene glycol and 2.5M NaCl in water. Solutions were incubated on ice or at 4 °C for 2 hours or overnight (respectively). Phage particles were isolated by pelleting at 17,000 x*g* at 4 °C for 20 minutes in a Sorvall RC 6+. The supernatant was decanted and the phage pellet was spun for an additional minute to collect remaining supernatant, which was then removed. The phage pellet was resuspended in approximately 1 mL of phosphate or Tris buffered saline, pH=7.4. Insoluble material was pelleted out of the phage solution at 17,000 x*g* for 5 minutes. Single-stranded, uridine-enriched DNA (ss dU DNA) was isolated from phage particles using a Qiagen M13 spin kit.

Kunkel Site-Directed Mutagenesis

Mutagenic primers were phosphorylated for 1 hour at 37 °C using T4 polynucleotide kinase. Phosphorylated primers were annealed to 1 μg of single stranded template DNA at a 10:1 molar ratio by incubating at 90 °C for 2 minutes, 50 °C for 3 minutes, and then 25 °C for 5 minutes. The annealed mixture was extended using T7 DNA polymerase in the presence of T4 DNA Ligase and 670 μM ATP and 330 μM dNTPs (each) at room temperature overnight. Reactions were cleaned up using a PCR spin kit and transformed into electrocompetent NEB-5alpha cells. This procedure typically yields 10^5 transformants with 40-80% mutation efficiency.

S.4. GFP Fluorescence-Based Sense Codon Reassignment Efficiency Assays

Superfolder green fluorescent protein (GFP) reporter plasmids (pGFP66xxx, where xxx specifies the codon at position 66) were co-transformed with vectors expressing the modified orthogonal translational components (pWB_Ultra-Tyr-yyy, where yyy indicates the anticodon on the tRNA) into SB3930 *E. coli*. After overnight growth, colonies were picked into 200 µL LB media in a 96 well plate. Cells were grown to saturation (usually 12 hours) with shaking at 37 °C. Cells were diluted 10-fold into LB media with antibiotics to maintain the pWB_Ultra and pGFP plasmids and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for induction of aaRS and GFP reporter. Assays were performed in a Fluorotrac 200 clear bottom 96 well plate (Greiner 655096) and monitored in a BioTek Synergy H1 plate reader at 37 °C with continuous double orbital shaking. The optical density (OD600) and fluorescence of each well was measured every 20 minutes; optical density was measured at 600 nm, and fluorescence was measured with an excitation at 485 nm and detection at 515 nm with an 8 nm band pass. The relative fluorescence of each 200 µL culture was calculated by dividing the LB media-blanked fluorescence by the OD600. The 100% relative fluorescence unit (reported as fluorescence units per unit optical density at 600 nm, RFU) value for sense codon reassignment efficiency was defined by taking an average of three cultures expressing wild type sfGFP (pGFP66tat) in cells harboring a negative control translational machinery plasmid (pWB_Ultra-Tyr-CUA) to maintain a similar metabolic burden on the cell. Sense codon reassignment efficiency for each tRNA variant was calculated by dividing the individual RFU values from each of three colonies by the average 100% reference RFU value and then averaging the results.

S.5. Phenol:Chloroform Extraction of RNA

NEB 5-alpha cells harboring a pWB_Ultra vector were grown overnight with shaking at 37 °C in LB/spectinomycin (50 µg/mL) media. Cell cultures were diluted 10-fold in fresh media to an approximate OD 600 nm = 0.1) and grown with shaking at 37 °C. When cellular cultures reached an OD600 of 0.5, 4 mL of cell culture were harvested by centrifugation in a microcentrifuge at 17000 x*g* for 1 minute. The supernatant was decanted, and the cell pellets were frozen at -20 °C overnight. The following day, cell pellets were thawed and mixed with 200 µL of B-PER lysis reagent (Thermo Scientific) at room temperature for 30 minutes. 200 µL of 0.3 M NaOAc, 10 mM EDTA, pH 4.5 were added to the lysis reactions to ensure the pH was close to 4.5. 450 µL of water-buffered phenol:chloroform (5:1 ratio) was then added to the lysate and vortexed three times for 60 seconds, 60 seconds, and 30 seconds, with lysates held on ice for 60 seconds between each step. The layers were then separated by centrifugation at 15000 x*g* at 4 °C for 15 minutes. The aqueous layer containing RNA was transferred 2.5 volumes of 100% EtOH for precipitation of nucleic acids. The mixture was vortexed briefly and stored overnight at -20 °C. The nucleic acids were recovered by centrifugation at 15000 x*g* at 4 °C for 30 minutes. The supernatant was aspirated, and the pellet was allowed to air dry prior to resuspension in 100 µL of 1x DNAseI Buffer (Thermo Scientific). One unit of DNaseI was added, and the solution was incubated at 37 °C for 20 minutes. DNaseI was inactivated by phenol:chloroform extraction and ethanol precipitation as described above. RNA was resuspended in 20 µL of 0.3 M NaOAc, 10 mM EDTA, pH 4.5 and quantified using a BioTek Synergy H1 plate reader. Each RNA extraction yielded 12-20 µg of RNA.

Table S1: pWB_Ultra aaRS/tRNA Expression Vectors

aaRS/tRNA Plasmid	Anticodon	Codon Read via Watson-Crick Interactions	Use
pWB_Ultra-Tyr-AUG	AUG	CAU	Reassignment of His CAU codons to Tyr[a]
pWB_Ultra-Tyr-(XhoI)	N/A	N/A	Starting template for tRNA library
pWB_Ultra-Tyr-CUA	CUA	UAG	Vector for sense codon reassignment controls[b]. Used in combination with a GFP reporter with a sense codon at position 66.

a) Biddle, W., Schmitt, M. A., and Fisk, J. D. (2015) Evaluating sense codon reassignment with a simple fluorescence screen, *Biochemistry*, **54**, 7355-7364.
b) Chatterjee, A., Sun, S. B., Furman, J. L., Xiao, H., & Schultz, P. G. (2013). A versatile platform for single-and multiple-unnatural amino acid mutagenesis in *Escherichia coli*. *Biochemistry*, **52**, 1828-1837.

Table S2: Reporter Protein Plasmid Designations and Usage

Reporter Plasmid	Codon at Position 66	Canonically Encoded Amino Acid	Use
pGFP66tat	UAU	Tyr	Wild type GFP vector for 100% fluorescence reference
pGFP66cac	CAC	His	Vector for evaluating CAC codon discrimination
pGFP66cau	CAU	His	Vector for evaluating reassignment of CAU sense codon
pGFP66caa	CAA	Gln	Vector for evaluating CAA codon discrimination

Vector design and construction reported in: Biddle, W., Schmitt, M. A., and Fisk, J. D. (2015) Evaluating sense codon reassignment with a simple fluorescence screen, *Biochemistry* **54**, 7355-7364.

Table S3: Oligonucleotide Primers

Primer for focused anticodon loop library of the *M. jannaschii* tRNA$^{Opt}_{AUG}$

Primer code	Primer sequence	Primer name
JQ	CGG CGG ANT atg NNT CCG CAT GGC AGG GGT TCA AAT CC	MjtRNA ATG His Lib JQ

Primers for reverse transcription, amplification, and sequencing of *E. coli* tRNA^{Arg2} and *M. jannaschii* tRNA$^{Opt}_{AUG}$ variants

Primer code	Primer sequence	Primer name
LW	GAC ACG GTA CCA CAC AAC TGG GCA ACG CAA CCT AGC TAA TGG TGC ATC CGG GAG GAT TCG	RT-PCR tRNA-Arg2 LW
LX	GAC ACG GTA CCA CAC AAC TGG	Wolf[a] Arg2 Primer LX
LY	GCA TCC GTA GCT CAG CTG G	Wolf[a] Arg3 Primer LY
LZ	GAC ACG GTA CCA CAC AAC TGG GCA ACG CAA CCT AGC TAA TGG TCC GGC GGA GGG GAT TTG	RT-PCR tRNA_Opt LZ
CC	GGC GTA TCT GCG CAG TAA GAT GCG CCC CGC ATT CCG GCG GTA GTT CAG CAG GGC AGA ACG	tRNA TBIO CC

a) Wolf, J., Gerber, A. P., and Keller, W. (2002) TadA, an essential tRNA-specific adenosine deaminase from Escherichia coli, *Embo Journal* **21**, 3841-3851.

Figure S1

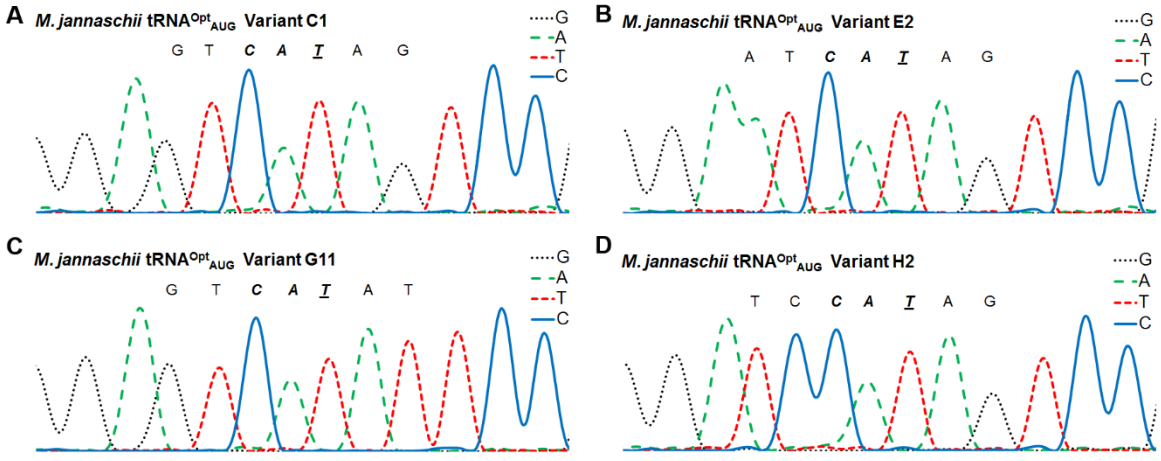

Figure S1. Sequence traces of the additional reverse transcribed *M. jannaschii* tRNA$^{Opt}_{AUG}$ variants isolated from the tRNA anticodon loop library. The portion of the chromatogram that corresponds to the tRNA anticodon sequence is italicized and bolded. In all cases the DNA encoded A34 is not modified to inosine.

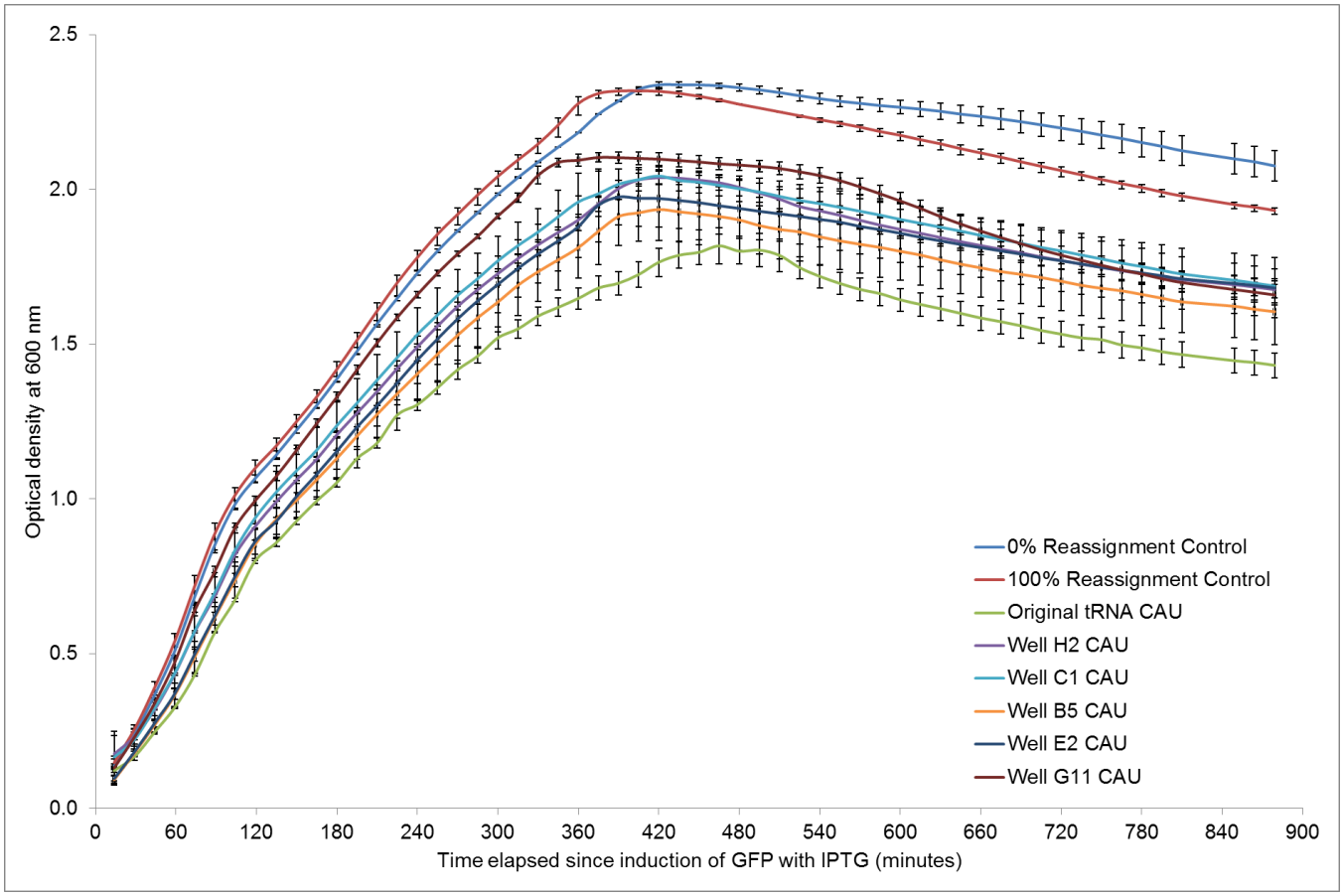

Figure S2: Optical density at 600 nm (OD_{600}) versus time profile for the *M. jannaschii* $tRNA^{Opt}_{AUG}$ variants evaluated in this manuscript. The original tRNA variant in which only the anticodon was changed exhibits the least carrying capacity. Each of the variants that result in improved discrimination between the CAU and CAC codons exhibit improved growth profiles more similar to those of the control systems.