

Figure 2c

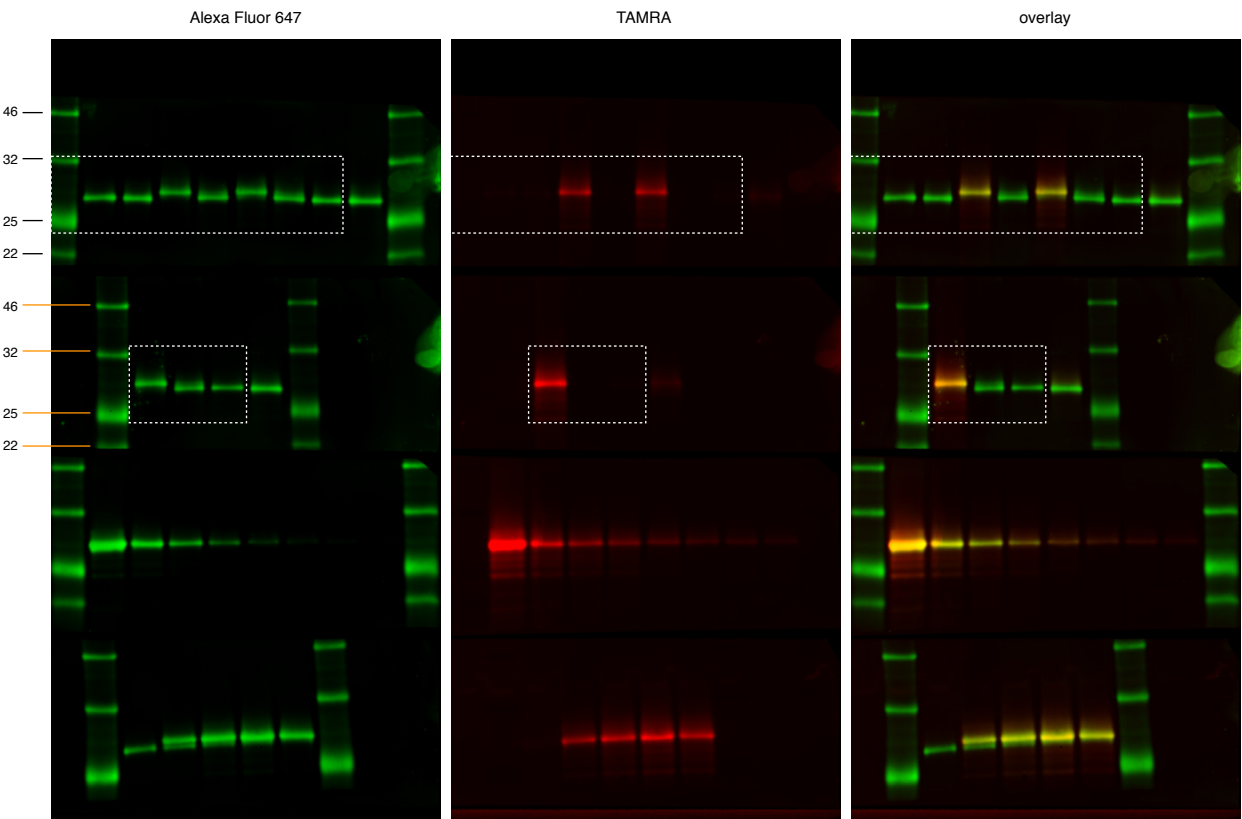
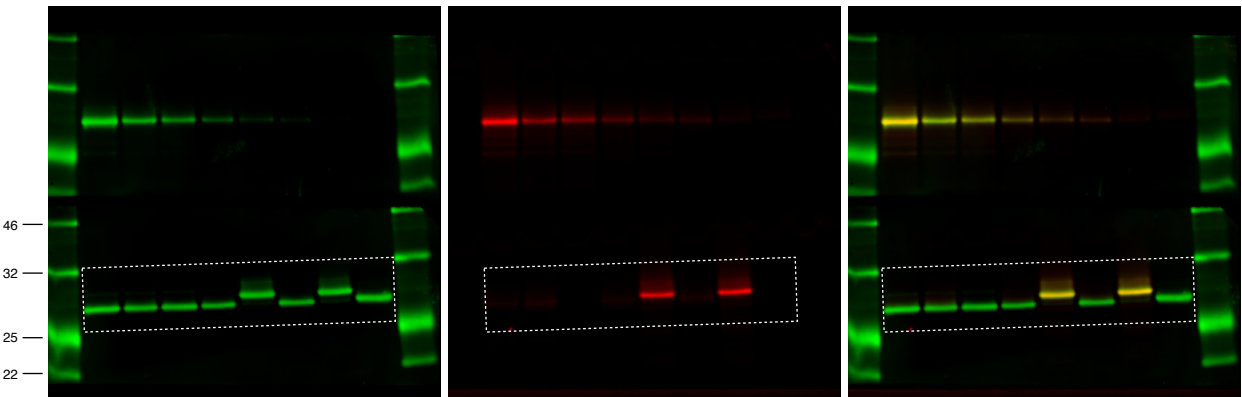
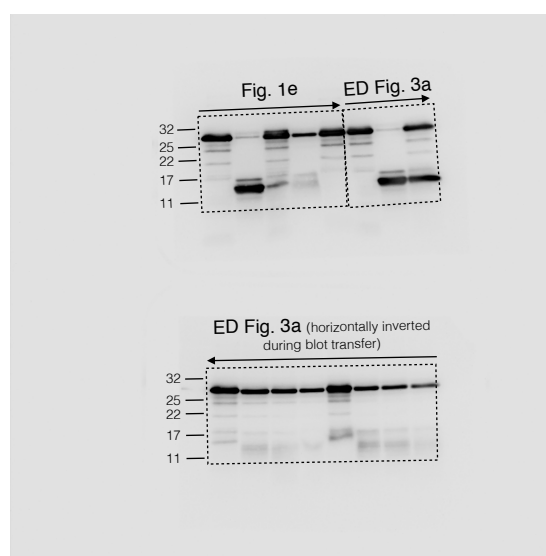


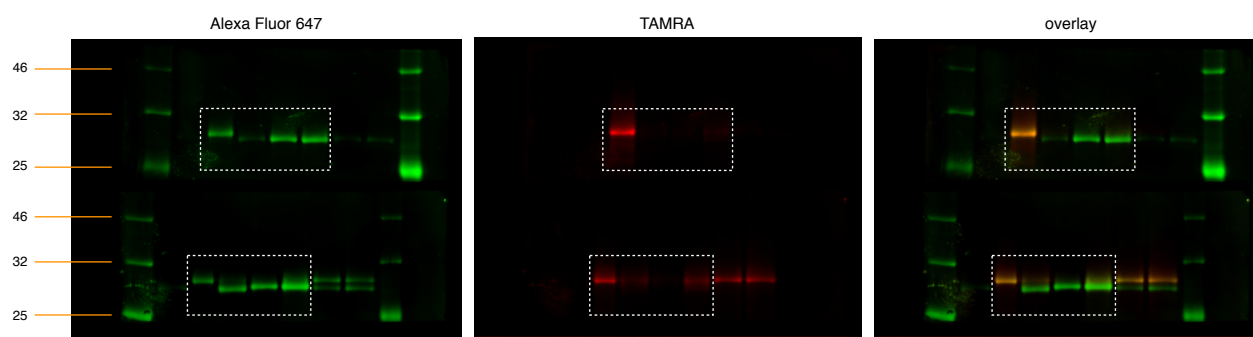
Figure 3b



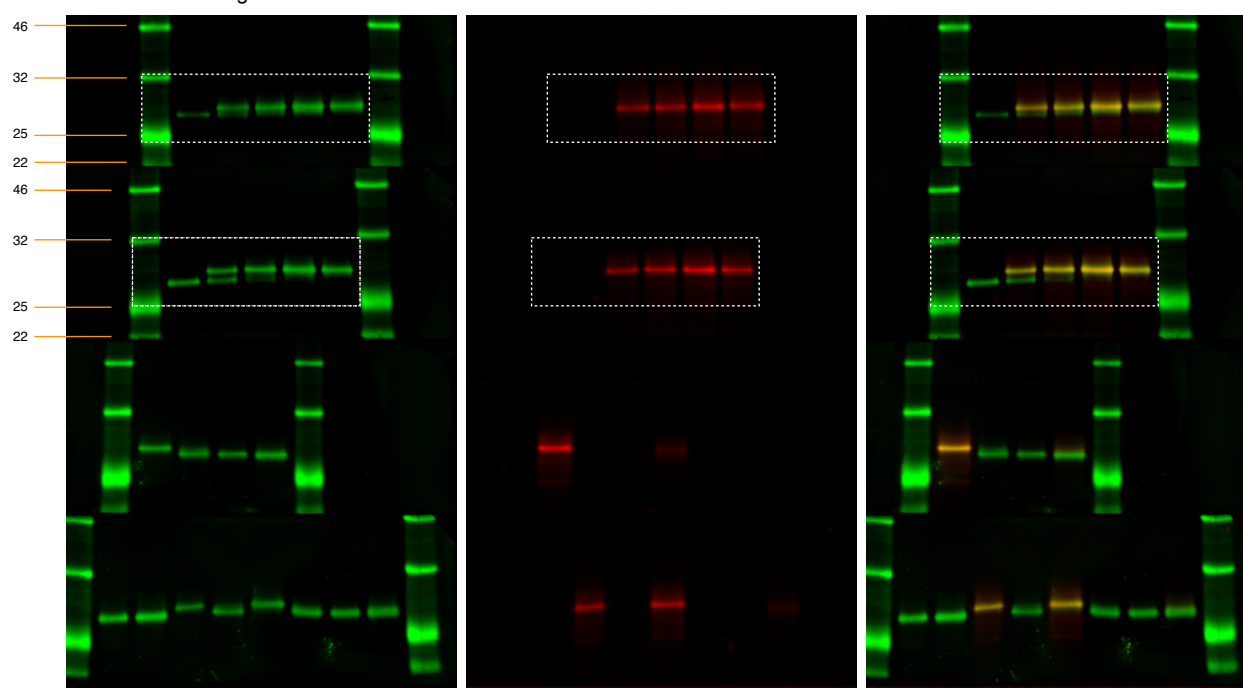
Supplementary Figure 1 | Uncropped scans with size marker indicators. Contiguous Alexa Fluor 647 and TAMRA images are each one simultaneous scan of multiple blots in parallel



Extended Data Figure 4a



Extended Data Figure 5a



Supplementary Figure 1 | Uncropped scans with size marker indicators. Contiguous Alexa Fluor 647 and TAMRA images are each one simultaneous scan of multiple blots in parallel

Supplementary Table 1: Plasmids used in this study

Annotated sequences (denoted by their respective GenBank accession nos.) are provided in Supplementary Notes for the sequences of synthetase expression plasmids and the prototypical Golden Gate destination plasmid for each plasmid class, as well as for a natural assembled plasmid example

Plasmid	GenBank Accession No.	Application	Relevant Properties
Synthetase Plasmids			
pGEX-empty TetR	MF996900	empty control plasmid; provides TetR in Ser incorporation experiments	P_{bla} - <i>tetR</i> P_{lacIq} - <i>lacI</i> amp ^R ori(pMB1)
pGEX-MbPylRS TetR	MF996901	PylRS expression plasmid	P_{lac} - <i>lacO</i> -PylRS P_{bla} - <i>tetR</i> P_{lacIq} - <i>lacI</i> amp ^R ori(pMB1)
pGEX-MjpAzFRS TetR	MF996902	$pAzFRS$ expression plasmid	P_{lacUV5} - <i>lacO</i> - $pAzFRS$ P_{bla} - <i>tetR</i> P_{lacIq} - <i>lacI</i> amp ^R ori(pMB1)
sfGFP Plasmids			
p[sfGFP(gg) ¹⁵¹]	MF996903	Golden Gate destination plasmid for sfGFP expression	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (Bsal-KpnI-Bsal) ¹⁵¹ -StrepTagII-T ₇ zeo ^R ori(p15A)
p[sfGFP(AGT) ¹⁵¹]	MF996904	natural sfGFP expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (AGT) ¹⁵¹ -StrepTagII-T ₇ zeo ^R ori(p15A)
p[sfGFP(TAC) ¹⁵¹]		natural sfGFP expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (TAC) ¹⁵¹ -StrepTagII-T ₇ zeo ^R ori(p15A)
p[sfGFP(TAG) ¹⁵¹]		natural sfGFP expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (TAG) ¹⁵¹ -StrepTagII-T ₇ zeo ^R ori(p15A)
p[sfGFP(ATC) ¹⁵¹]		natural sfGFP expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (ATC) ¹⁵¹ -StrepTagII-T ₇ zeo ^R ori(p15A)
p[sfGFP(AAC) ¹⁵¹]		natural sfGFP expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (AAC) ¹⁵¹ -StrepTagII-T ₇ zeo ^R ori(p15A)
p[sfGFP(ACC) ¹⁵¹]		natural sfGFP expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (ACC) ¹⁵¹ -StrepTagII-T ₇ zeo ^R ori(p15A)
p[sfGFP(AGC) ¹⁵¹]		natural sfGFP expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (AGC) ¹⁵¹ -StrepTagII-T ₇ zeo ^R ori(p15A)
p[sfGFP(GTC) ¹⁵¹]		natural sfGFP expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (GTC) ¹⁵¹ -StrepTagII-T ₇ zeo ^R ori(p15A)
p[sfGFP(GAC) ¹⁵¹]		natural sfGFP expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (GAC) ¹⁵¹ -StrepTagII-T ₇ zeo ^R ori(p15A)
p[sfGFP(GCC) ¹⁵¹]		natural sfGFP expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (GCC) ¹⁵¹ -StrepTagII-T ₇ zeo ^R ori(p15A)
p[sfGFP(GGC) ¹⁵¹]		natural sfGFP expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (GGC) ¹⁵¹ -StrepTagII-T ₇ zeo ^R ori(p15A)
sfGFP and tRNA^{Ser} Plasmids			
p[sfGFP(gg) ¹⁵¹ tRNA ^{Ser} (gg)]	MF996905	Golden Gate destination plasmid for sfGFP and tRNA ^{Ser} expression	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (Bsal-KpnI-Bsal) ¹⁵¹ -StrepTagII-T ₇ P_{T7} - <i>lacO</i> - <i>serT</i> (Bsal-KpnI-Bsal)-T ₇ zeo ^R ori(p15A)
p[sfGFP(TAG) ¹⁵¹ tRNA ^{Ser} (CTA)]	MF996906	natural sfGFP and tRNA ^{Ser} expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (TAG) ¹⁵¹ -StrepTagII-T ₇ P_{T7} - <i>lacO</i> - <i>serT</i> (CTA)-T ₇ zeo ^R ori(p15A)
p[sfGFP(gg) ¹⁵¹ tRNA ^{Ser} (GAT)]	MF996907	Golden Gate destination plasmid for sfGFP and tRNA ^{Ser} expression	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (Bsal-KpnI-Bsal) ¹⁵¹ -StrepTagII-T ₇ P_{T7} - <i>lacO</i> - <i>serT</i> (GAT)-T ₇ zeo ^R ori(p15A)
p[sfGFP(gg) ¹⁵¹ tRNA ^{Ser} (GGT)]		Golden Gate destination plasmid for sfGFP and tRNA ^{Ser} expression	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (Bsal-KpnI-Bsal) ¹⁵¹ -StrepTagII-T ₇ P_{T7} - <i>lacO</i> - <i>serT</i> (GGT)-T ₇ zeo ^R ori(p15A)
p[sfGFP(gg) ¹⁵¹ tRNA ^{Ser} (GCT)]		Golden Gate destination plasmid for sfGFP and tRNA ^{Ser} expression	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (Bsal-KpnI-Bsal) ¹⁵¹ -StrepTagII-T ₇ P_{T7} - <i>lacO</i> - <i>serT</i> (GCT)-T ₇ zeo ^R ori(p15A)
p[sfGFP(gg) ¹⁵¹ tRNA ^{Ser} (GTT)]		Golden Gate destination plasmid for sfGFP and tRNA ^{Ser} expression	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (Bsal-KpnI-Bsal) ¹⁵¹ -StrepTagII-T ₇ P_{T7} - <i>lacO</i> - <i>serT</i> (GTT)-T ₇ zeo ^R ori(p15A)
sfGFP and tRNA^{Pyl} Plasmids			
p[sfGFP(gg) ¹⁵¹ tRNA ^{Pyl} (gg)]	MF996908	Golden Gate destination plasmid for sfGFP and tRNA ^{Pyl} expression	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (Bsal-KpnI-Bsal) ¹⁵¹ -StrepTagII-T ₇ P_{T7} - <i>lacO</i> -tRNA ^{Pyl} (Bsal-KpnI-Bsal)-T ₇ zeo ^R ori(p15A)
p[sfGFP(TAG) ¹⁵¹ tRNA ^{Pyl} (CTA)]	MF996909	natural sfGFP and tRNA ^{Pyl} expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (TAG) ¹⁵¹ -StrepTagII-T ₇ P_{T7} - <i>lacO</i> -tRNA ^{Pyl} (CTA)-T ₇ zeo ^R ori(p15A)
p[sfGFP(TAC) ¹⁵¹ tRNA ^{Pyl} (gg)]	MF996910	Golden Gate destination plasmid for sfGFP and tRNA ^{Pyl} expression	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (TAC) ¹⁵¹ -StrepTagII-T ₇ P_{T7} - <i>lacO</i> -tRNA ^{Pyl} (Bsal-KpnI-Bsal)-T ₇ zeo ^R ori(p15A)
sfGFP and tRNA^{pAzF} Plasmids			
p[sfGFP(gg) ¹⁵¹ tRNA ^{pAzF} (gg)]	MF996911	Golden Gate destination plasmid for sfGFP and tRNA ^{pAzF} expression	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (Bsal-KpnI-Bsal) ¹⁵¹ -StrepTagII-T ₇ P_{T7} - <i>lacO</i> -tRNA ^{pAzF} (Bsal-KpnI-Bsal)-T ₇ zeo ^R ori(p15A)
p[sfGFP(TAG) ¹⁵¹ tRNA ^{pAzF} (CTA)]	MF996912	natural sfGFP and tRNA ^{pAzF} expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (TAG) ¹⁵¹ -StrepTagII-T ₇ P_{T7} - <i>lacO</i> -tRNA ^{pAzF} (CTA)-T ₇ zeo ^R ori(p15A)
p[sfGFP(TAC) ¹⁵¹ tRNA ^{pAzF} (CTA)]		natural sfGFP and tRNA ^{pAzF} expression plasmid	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (TAC) ¹⁵¹ -StrepTagII-T ₇ P_{T7} - <i>lacO</i> -tRNA ^{pAzF} (CTA)-T ₇ zeo ^R ori(p15A)
p[sfGFP(TAC) ¹⁵¹ tRNA ^{pAzF} (gg)]	MF996913	Golden Gate destination plasmid for sfGFP and tRNA ^{pAzF} expression	P_{T7} - <i>tetO</i> - <i>sfGFP</i> (TAC) ¹⁵¹ -StrepTagII-T ₇ P_{T7} - <i>lacO</i> -tRNA ^{pAzF} (Bsal-KpnI-Bsal)-T ₇ zeo ^R ori(p15A)

Supplementary Table 2: Primers used in this study

Primers for UBP-containing Golden Gate inserts

Italics denote the BsaI recognition sequence; underline denotes the BsaI restriction site overhang sequence
Asterisk (*) denotes primers that were also used for the biotin shift assay
All primers were used with an annealing temperature of 54°C

Primer	Application	Sequence (5'→3')
YZ73	<i>sfGFP</i> position 151 insert F	ATGGGTCTCACACA <u>AA</u> ACTCGAGTACAACTTTAACTCACAC
YZ74	<i>sfGFP</i> position 151 insert R	ATGGGTCTCGATTCCATTCTTTTGTGTCTGC
YZ401*	<i>E. coli</i> tRNA ^{Ser} insert F	ATTGGTCTCGGCCGAGCGGTTGAAGGCAC
YZ403*	<i>E. coli</i> tRNA ^{Ser} insert R	ATTGGTCTCTCTGGAACCCCTTTCGGGTCG
YZ435*	<i>M. mazei</i> tRNA ^{Pyl} insert F	ATGGGTCTCGAAACCTGATCATGTAGATCGAACGG
YZ436*	<i>M. mazei</i> tRNA ^{Pyl} insert R	ATGGGTCTCATCTAACCCTGGCTGAACGG
YZ85	<i>M. jannaschii</i> tRNA ^{pAzF} insert F	ATGGGTCTCCGGTAGTTTCAGCAGGGCAGAACG
YZ86	<i>M. jannaschii</i> tRNA ^{pAzF} insert R	ATGGGTCTCGAGGGGATTGAACCCCTGCCATG

Biotin shift assay primers

All primers were used with an annealing temperature of 54°C

Primer	Application	Sequence (5'→3')
JP356	tRNA ^{pAzF} and tRNA ^{Ser} external F	GATAACAATTCCCCTCTAGAAAAATCATT
JP357	tRNA ^{pAzF} and tRNA ^{Ser} external R	GCTTATGCAAAAAGCCTGCTCGTTTCAG
YZ351	<i>sfGFP</i> position 151 internal F	CTCGAGTACAACTTTAACTCACAC
YZ352	<i>sfGFP</i> position 151 internal R	GATTCCATTCTTTTGTGTCTGC

Golden Gate insert template oligonucleotides

X denotes dNaM; the sequences corresponding to *sfGFP* position 151 codons and tRNA anticodons are underlined
sfGFP oligonucleotides correspond to the sense strand; tRNA oligonucleotides correspond to the antisense strand

Name	Sequence Type	Sequence (5'→3')
GFP151(TAG)	<i>sfGFP</i> position 151 codon	CTCGAGTACAACTTTAACTCACACAATGTATAGATCACGGCAGACAAACAAAAGAATGGAATC
GFP151(TAC)	<i>sfGFP</i> position 151 codon	CTCGAGTACAACTTTAACTCACACAATGTATACATCACGGCAGACAAACAAAAGAATGGAATC
GFP151(AXC)	<i>sfGFP</i> position 151 codon	CTCGAGTACAACTTTAACTCACACAATGTAA <u>XC</u> ATCACGGCAGACAAACAAAAGAATGGAATC
GFP151(GXC)	<i>sfGFP</i> position 151 codon	CTCGAGTACAACTTTAACTCACACAATGTAG <u>XC</u> ATCACGGCAGACAAACAAAAGAATGGAATC
ESer(GYT)	<i>E. coli</i> tRNA ^{Ser} anticodon	CTCTGGAACCCCTTTCGGGTCGCCGGTTTAA <u>XC</u> AGACCGGTGCCTTCAACCGCTCGGC
Mm(CTA)	<i>M. mazei</i> tRNA ^{Pyl} anticodon	GAATCTAACCCTGGCTGAACGGATTTAGAGTCCGTTTCGATCTACATGATCAGG
Mm(GYT)	<i>M. mazei</i> tRNA ^{Pyl} anticodon	GAATCTAACCCTGGCTGAACGGATTAA <u>XC</u> AGTCCGTTTCGATCTACATGATCAGG
Mm(GYC)	<i>M. mazei</i> tRNA ^{Pyl} anticodon	GAATCTAACCCTGGCTGAACGGATTG <u>XC</u> AGTCCGTTTCGATCTACATGATCAGG
Mj(CTA)	<i>M. jannaschii</i> tRNA ^{pAzF} anticodon	GATTTGAACCCCTGCCATGCGGATTTAGAGTCCGCCGTTCTGCCCTGCTGAA
Mj(GYT)	<i>M. jannaschii</i> tRNA ^{pAzF} anticodon	GATTTGAACCCCTGCCATGCGGATTAA <u>XC</u> AGTCCGCCGTTCTGCCCTGCTGAA