## **Supporting Information**

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**Fig. S1.** Dependence on EF-Tu of dipeptide synthesis from fMet-tRNA<sup>fMet</sup> and *N*-methyl-Phe-tRNA<sup>Phe</sup> at Phe codon UUC. Diamonds, kinetics of dipeptide synthesis in the absence of EF-Tu. Triangles, kinetics of dipeptide synthesis in the presence of EF-Tu (taken from Fig. 2*D* for comparative purposes).



**Fig. S2.** Reversed-phase HPLC elution profiles of dipeptide products from  $[^{3}H]fMet-tRNA^{fMet}$  and  $tRNA^{PheB}$  charged with Phe (*A*), *N*-methyl-Phe (*B*), and *N*-butyl-Phe (*C*) using Phe codon UUC. Translation reactions were carried out for 0.15 s (*A*), 1,800 s = 30 min (*B*), and 1,800 s (*C*), and samples for HPLC were prepared as described in *Materials and Methods*. Counts per min (CPM) are plotted against elution time (min). (*A*–*C*) Elution for min 0–20 was isocratic with 42% methanol/0.1% trifluoroacetic acid. (*C*) Elution for min 20–25 was at 50% methanol/0.1% trifluoroacetic acid, and elution for min 25–30 was at 74% methanol/0.1% trifluoroacetic acid.

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## Table S1. Summary of dipeptide synthesis rates

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Aminoacyl-tRNA	Codon	$k_{ m dip}$ , s $^{-1}$	$k_{ m GTP}$ , s $^{-1}$	$k_{ m acc, pep},{ m s}^{-1}$
Phe-tRNA <sup>Phe</sup>	UUU	51 (±5)	129 (±10)	84 (±14)
Phe-tRNA <sup>Phe</sup>	UUC	54 (±5)	130 (±10)	92 (±15)
Phe-tRNA <sup>Bulk</sup>	UUC	49 (±4)	112 (±10)	87 (±14)
Phe-tRNA <sup>PheB</sup>	UUC	41 (±4)	77 (±4)	90 (±19)
<i>N</i> -methyl-Phe-tRNA <sup>PheB</sup>	UUC	0.005 (±0.0003)	19 (±3)	0.005 (±0.0003)
N-butyl-Phe-tRNA <sup>PheB</sup>	UUC	Undetectable	15 (±7)	Undetectable
Ala-tRNA <sup>PheB</sup>	UUC	27 (±5)	76 (±8)	42 (±12)
Pro-tRNA <sup>PheB</sup>	UUC	1.75 (±0.2)	62 (±12)	1.8 (±0.2)
Pro-tRNA <sup>Bulk</sup>	CCG	16 (±2)	80 (±9)	20 (±3)
Pro-tRNA <sup>Bulk</sup>	CCA	15 (±2)	53 (±8)	21 (±4)
Pro-tRNA <sup>Bulk</sup>	CCU	14 (±2)	42 (±8)	21 (±4)
Pro-tRNA <sup>Bulk</sup>	ссс	8.6 (±1.2)	20 (±2)	15 (±4)

Data are expressed as rates for GTP hydrolysis ( $k_{GTP}$ ) and dipeptide synthesis from fMet-tRNA<sup>fMet</sup> ( $k_{dip}$ ). The rates are calculated from Table 1 by  $k = 1/\tau$ . Thus,  $k_{acc,pep} = 1/(1/k_{dip} - 1/k_{GTP})$ .

## Table S2. Complete sequences of mRNAs

PNAS PNAS

mRNA	Sequence		
mMF <sub>UUC</sub> TI	gggaauucgggcccuuguuaacaauuaaggagguauauc		
	AUG UUC ACG AUU uaauugcagaaaaaaaaaaaaaaaaaaaaaaaaaaa		
mMF <sub>UUU</sub> TI	gggaauucgggcccuuguuaacaauuaaggagguauauc		
	AUG UUU ACG AUU uaauugcagaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa		
mMP <sub>CCA</sub> F	gggaauucgggcccuuguuaacaauuaaggagguauauc		
	AUG CCA UUC uaaucugcagaaaaaaaaaaaaaaaaagcg		
mMP <sub>ccc</sub> F	gggaauucgggcccuuguuaacaauuaaggagguauauc		
	AUG CCC UUC uaaucugcagaaaaaaaaaaaaaaaaagcg		
mMP <sub>ccg</sub> F	gggaauucgggcccuuguuaacaauuaaggagguauauc		
	AUG CCG UUC uaaucugcagaaaaaaaaaaaaaaaaagcg		
mMP <sub>ccu</sub> F	gggaauucgggcccuuguuaacaauuaaggagguauauc		
	AUG CCU UUC uaaucugcagaaaaaaaaaaaaaaaaagcg		

All mRNAs were prepared by transcription with T7 RNA polymerase, purified by binding to an oligo(dT) column, and have the same upstream sequence including a strong Shine–Dalgarno ribosome-binding sequence (uaaggaggu). Sense codons are in capitals in the sequences. Translated elongator codons are in subscripts in the mRNA names.