Methylation of *E. coli* Class I Release Factors Increases the Accuracy of Translation Termination *in vitro*

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Abbreviations: RF - release factor, RC - release complex

ABSTRACT

Ribosomal protein synthesis (translation) is a highly accurate process. Translation termination, in particular, must be accurate to prevent truncated proteins. How this accuracy is achieved is not fully understood in all its details. Using an *E. coli in vitro* system, I explore novel mechanisms that contribute to the high accuracy of translation termination. By comparing the Michaelis-Menten parameters of methylated and non-methylated release factors on cognate and non-cognate codons. Post-translational methylation of a strictly conserved GGQ motif in class I release factors increases the accuracy of termination by up to 5-fold. This happens by increasing both the maximum rate of peptide release (k_{cat}) and Michaelis-Menten constant (K_M). Further, I demonstrate here that a non-methylated release factor acts like an uncompetitive inhibitor of enzyme reactions. Overall, this study shows that the methylation of class I release factors is a novel mechanism contributing to highly accurate translation termination.

15 **INTRODUCTION**

16 Translation termination is an essential component of protein production. mRNA
17 translation is terminated when a stop codon is translocated into the ribosomal A site.
18 This new configuration is now called a release complex (RC). The subsequent binding
19 of the class I release factor induces peptide release. In nearly all life forms, the codons
20 UAA, UAG, and UGA are used as termination signals, with some exceptions (Ivanova
21 et al., 2014). In bacteria, the class I release factors are RF1 and RF2. Both class I
22 release factors possess a strictly conserved glycine-glycine-glutamine (GGQ) motif,
23 which is essential for the ester bond hydrolysis by which the peptide is released from
24 the tRNA.

25 Stop codon recognition is highly accurate *in vitro* (Freistroffer et al., 2000) and *in vivo*
26 (Jørgensen et al., 1993), but our understanding of how this accuracy is achieved is
27 still rudimentary. The accuracy of a reaction is defined as the ratio of the efficiency of
28 a cognate to a non-cognate reaction (Freistroffer et al., 2000). The efficiency is
29 determined by the ratio of the maximum rate of the reaction (k_{cat}) to the concentration
30 at half k_{cat} (K_M). When the ratio of cognate and non-cognate reaction is one, accuracy
31 is non-existent, meaning no discrimination between the cognate and non-cognate
32 substrate are observable.

33 Methylation of the glutamine in the GGQ motif has been shown to increase k_{cat}
34 (Indrisiunaite et al., 2015). Methylation happens via the hemK protein, which modifies
35 glutamine 253 (*E. coli* numbering) to N^5-methyl-glutamine (Heurgue-Hamard et al.,
36 2002). However, its effect on K_M is unknown.

37 Highly conserved recognition motifs of RF1 and RF2 are located in the vicinity of the
38 stop codons, but about 75Å distant from the catalytic center (Korostelev, 2011). These
39 motifs (PxT in RF1 and SPF in RF2) were thought to be responsible for the specificity

40 of stop codon recognition by the release factors and therefore named 'tripeptide
41 anticodons', analogous to the anticodons of tRNAs (Ito et al., 1996, 2000).
42 Class II release factor RF3 was shown to affect accuracy partly (Freistroffer et al.,
43 2000). Further, it was shown that depletion of the ribosomal protein L11 from an RC
44 increases the accuracy of translation termination with RF1 but not RF2 (Bouakaz et
45 al., 2006). The accuracy of translation termination is mostly thought to be due to the
46 extensive interaction network between class I release factor, stop codon, and rRNA
47 (Sund et al., 2010).
48 The strictly conserved GGQ motif is essential for ester bond hydrolysis, as shown by
49 studies where mutations within the GGQ motif disrupted translation termination *in vitro*
50 (Zavialov et al., 2002) and *in vivo* (Frolova et al., 1999; Seit-Nebi et al., 2001).
51 However, in *E. coli* different mechanisms seem to be at play for several reasons. The
52 conserved methylation of the GGQ motif glutamine to N^5-methyl-glutamine is not
53 essential in *E. coli*, as the responsible enzyme for methylation RF1/RF2, hemK, is not
54 essential for cell viability (Heurgue-Hamard et al., 2002; Pannekoek et al., 2005). Also,
55 release factors where the GGQ motif is altered to GGA are still able to induce
56 translation termination *in vitro* (Shaw and Green, 2007; Zavialov et al., 2002). On the
57 other hand, methylation of the class I release factors is essential for cell viability in
58 minimal media (Mora et al., 2007). As mentioned earlier, methylation increases the
59 maximum rate of peptide release (k_{cat}) (Indrisiunaite et al., 2015), but the magnitude
60 of the k_{cat} increase was shown to depend on the nature of the amino acid on the tRNA
61 occupying the P site (Pierson et al., 2016).
62 The actual ester bond hydrolysis occurs via a coordination of a nucleophilic hydroxide
63 molecule by the GGQ motif into the PTC. Crystal structures of class I release factors

64 indicate a difference between the positioning of the methylated and non-methylated
65 glutamine residue within the PTC (Pierson et al., 2016).
66 Although RF1 and RF2 share high sequence and structural similarity, they have
67 different codon specificities. RF1 recognizes the stop codons UAA and UAG, whereas
68 RF2 recognizes the stop codons UAA and UGA (Scolnick et al., 1968).

RESULTS & DISCUSSION

In this study, I investigated the methylation of the GGQ glutamine as an additional factor that might enhance the accuracy of translation termination *in vitro*. The accuracy of a reaction is defined as the ratio of the efficiency (k_{cat} / K_M) of a cognate to a non-cognate reaction (Freistroffer et al., 2000). When the ratio of cognate and non-cognate reaction is one, accuracy is non-existent, meaning no discrimination between the cognate and non-cognate substrate are observable. I systematically determined the k_{cat} and K_M parameters for release factors on various purified release complexes (Korkmaz and Sanyal, 2017) containing cognate and non-cognate codons in A site and compared the resulting accuracy with the reactions involving methylated (mRF1 or mRF2) and non-methylated (RF1 or RF2) release factors (Table 1 and Figure 1 and 2). UAA was used as the cognate codon in all experiments, while UAG and UGA were used as the non-cognate substrates for RF1 and RF2 respectively since these codons allow discrimination similar to non-cognate sense codons (Freistroffer et al., 2000).

The determined cognate k_{cat} parameters were in agreement with previous data for non-methylated release factors (Bouakaz et al., 2006; Freistroffer et al., 2000) and also with recent studies that used methylated release factors (Indrisiunaite et al., 2015; Pierson et al., 2016). I observed a 4.5-fold increase in the maximum rate of peptide release for mRF1 on its cognate UAA codon and a 5-fold increase in the maximum rate of tritium-labeled fMet (^{3H}fMet) release on the non-cognate codon UGA. However, it should be noted that for the cognate reactions, the K_M values for reactions with either mRF1 or RF1 were below the detection limit of 5 nM. Thus, the real difference in K_M might be much larger than what could be measured in these experiments. In Table 1 the estimated K_M parameters are listed. The K_M value form RF1 on UGA decreased by 3-fold, from 164 nM to 48 nM, when methylation was missing. These resulted in an

94 accuracy of 25 for RF1 on UGA. When RF1 was methylated, the accuracy increased
95 to 90 (an increase of over 3.5-fold) (Table 1).
96 The effect of methylation on the translation termination accuracy was even more
97 pronounced with RF2 on the non-cognate UAG codon. With methylation, the k_{cat}
98 increased 2.6-fold for the non-cognate substrate and nearly 16-fold for the cognate
99 substrate (UAA). The accuracy of translation termination increased 5-fold, from 13 to
100 68, when RF2 was methylated.
101 As mentioned earlier, it is not surprising that the maximum rate of ^{3H}fMet release
102 increased in all cases; however, unexpectedly, the K_M parameters were also affected
103 by release factor methylation. For RF1 and mRF1, the change in K_M (Table 1) could
104 not be unambiguously determined in the experimental setup as both values were
105 below the detection limit, but an approximately 3-fold difference in K_M was observed
106 for the non-cognate reading of UGA; methylation of RF1 resulted in an increase of the
107 K_M value. More pronounced changes in K_M were observed for RF2 and mRF2. For
108 cognate stop codon recognition, a nearly 5-fold increase in K_M was observed when
109 RF2 was methylated (26 nM for RF2 and 123 nM for mRF2); a 4-fold increase was
110 seen in the case of the non-cognate substrate UAG (251 nM for RF2 and 1045 nM for
111 mRF2).
112 When hemK is knocked out, cells have compromised fitness, yet they are viable (Mora
113 et al., 2007). The knockout of hemK consequently leads to the lack of methylation in
114 class I RFs and as shown here. The protein hemK becomes essential when cells are
115 grown in minimal media (Mora et al., 2007). In the light of the data presented here,
116 one could explain this phenotype by the fact of the lower accuracy of RFs than the
117 slowed down peptide release. In minimal media, ribosomes are likely to pause on

118 codons due to the amino acid limitation (Li et al., 2012). Inaccurate RFs could bind to
119 these complexes and induce premature termination.
120 Interestingly, the Lineweaver-Burk plots of the methylated and non-methylated release
121 factor kinetics data revealed that the non-methylated release factors behave similarly
122 to uncompetitive inhibitors in enzyme reactions (Figure 3). However, the mechanism
123 of the inhibition or details of any mechanistic similarity to uncompetitive enzyme
124 inhibition remains obscure.
125 Overall, I herein presented evidence that methylation of class I release factors
126 contribute to the accuracy of translation termination. This is surprising by the fact that
127 a post-translational modification, far from the initial binding site, effects the binding
128 constant K_M.
129 Further studies, unraveling the potential *in vivo* aspects of this finding are imminent,
130 especially in light of the ribosome rescue phenomenon. This study demonstrates that
131 release factor methylation plays an additional biological role beyond the known
132 increase of the maximum rate of peptide release. This previously unknown variable
133 can explain how the outstanding accuracy of translation termination is achieved in the
134 absence of any proofreading steps.

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CONFLICT OF INTEREST

No conflict of interest is stated.

149 **REFERENCES**

150 Bouakaz, L., Bouakaz, E., Murgola, E.J., Ehrenberg, M., and Sanyal, S. (2006). The
151 Role of Ribosomal Protein L11 in Class I Release Factor-mediated Translation
152 Termination and Translational Accuracy. J. Biol. Chem. *281*, 4548–4556.

153 Freistroffer, D.V., Kwiatkowski, M., Buckingham, R.H., and Ehrenberg, M. (2000).
154 The accuracy of codon recognition by polypeptide release factors. Proc Natl Acad
155 Sci USA *97*, 2046–2051.

156 Frolova, L.Y., Tsivkovskii, R.Y., Sivolobova, G.F., Oparina, N.Y., Serpinsky, O.I.,
157 Blinov, V.M., Tatkov, S.I., and Kisselev, L.L. (1999). Mutations in the highly conserved
158 GGQ motif of class 1 polypeptide release factors abolish ability of human eRF1 to
159 trigger peptidyl-tRNA hydrolysis. RNA *5*, 1014–1020.

160 Heurgue-Hamard, V., Champ, S., Engstrom, A., Ehrenberg, M., and Buckingham,
161 R.H. (2002). The hemK gene in Escherichia coli encodes the N5-glutamine
162 methyltransferase that modifies peptide release factors. EMBO J *21*, 769–778.

163 Indrisiunaite, G., Pavlov, M.Y., Heurgué-Hamard, V., and Ehrenberg, M. (2015). On
164 the pH Dependence of Class-1 RF-Dependent Termination of mRNA Translation. J.
165 Mol. Biol. *427*, 1848–1860.

166 Ito, K., Ebihara, K., Uno, M., and Nakamura, Y. (1996). Conserved motifs in
167 prokaryotic and eukaryotic polypeptide release factors: tRNA-protein mimicry
168 hypothesis. Proc. Natl. Acad. Sci. U. S. A. *93*, 5443–5448.

169 Ito, K., Uno, M., and Nakamura, Y. (2000). A tripeptide “anticodon” deciphers stop
170 codons in messenger RNA. Nature *403*, 680–684.

171 Ivanova, N.N., Schwientek, P., Tripp, H.J., Rinke, C., Pati, A., Huntemann, M., Visel,
172 A., Woyke, T., Kyrpides, N.C., and Rubin, E.M. (2014). Stop codon reassignments in
173 the wild. Science *344*, 909–913.

174 Jelenc, P.C., and Kurland, C.G. (1979). Nucleoside triphosphate regeneration
175 decreases the frequency of translation errors. Proc. Natl. Acad. Sci. *76*, 3174–3178.

176 Jørgensen, F., Adamski, F.M., Tate, W.P., and Kurland, C.G. (1993). Release Factor-
177 dependent False Stops are Infrequent in Escherichia coli. J. Mol. Biol. *230*, 41–50.

178 Korkmaz, G., and Sanyal, S. (2017). R213I mutation in release factor 2 (RF2) is one
179 step forward for engineering an omnipotent release factor in bacteria Escherichia
180 coli. J. Biol. Chem. *292*, 15134–15142.

181 Korostelev, A.A. (2011). Structural aspects of translation termination on the
182 ribosome. RNA N. Y. N *17*, 1409–1421.

183 Li, G.-W., Oh, E., and Weissman, J.S. (2012). The anti-Shine-Dalgarno sequence
184 drives translational pausing and codon choice in bacteria. Nature *484*, 538–541.

Mora, L., Heurgué-Hamard, V., de Zamaroczy, M., Kervestin, S., and Buckingham, R.H. (2007). Methylation of Bacterial Release Factors RF1 and RF2 Is Required for Normal Translation Termination in Vivo. J. Biol. Chem. *282*, 35638–35645.

Pannekoek, Y., Heurgue-Hamard, V., Langerak, A.A.J., Speijer, D., Buckingham, R.H., and van der Ende, A. (2005). The N5-Glutamine S-Adenosyl-L-Methionine-Dependent Methyltransferase PrmC/HemK in Chlamydia trachomatis Methylates Class 1 Release Factors. J Bacteriol *187*, 507–511.

Pierson, W.E., Hoffer, E.D., Keedy, H.E., Simms, C.L., Dunham, C.M., and Zaher, H.S. (2016). Uniformity of Peptide Release Is Maintained by Methylation of Release Factors. Cell Rep. *17*, 11–18.

Scolnick, E., Tompkins, R., Caskey, T., and Nirenberg, M. (1968). Release factors differing in specificity for terminator codons. Proc Natl Acad Sci USA *61*, 768–774.

Seit-Nebi, A., Frolova, L., Justesen, J., and Kisselev, L. (2001). Class-1 translation termination factors: invariant GGQ minidomain is essential for release activity and ribosome binding but not for stop codon recognition. Nucleic Acids Res *29*, 3982–3987.

Shaw, J.J., and Green, R. (2007). Two Distinct Components of Release Factor Function Uncovered by Nucleophile Partitioning Analysis. Mol. Cell *28*, 458–467.

Sund, J., Ander, M., and Aqvist, J. (2010). Principles of stop-codon reading on the ribosome. Nature *465*, 947–950.

Zavialov, A.V., Mora, L., Buckingham, R.H., and Ehrenberg, M. (2002). Release of Peptide Promoted by the GGQ Motif of Class 1 Release Factors Regulates the GTPase Activity of RF3. Mol. Cell *10*, 789–798.

209 **Figure & Tables**

210 **Table 1** Comparison of Michaelis-Menten parameters and accuracy measurements of
211 methylated and non-methylated class I release factors. The *E. coli* K12 genes
212 encoding RF1 (*prfA*) and RF2 (*prfB*) were already available as clones in the pTRC and
213 pET11a vectors, respectively. The final construct pET11a-prfB encoded a prfB^{T246A}
214 variant to enable the overexpression of an otherwise toxic RF2 (Uno et al., 1996). Both
215 constructs contained C-terminal His$_6$-tags. The release factor methyltransferase *hemK*
216 (gene: *prmC*), was available as a clone in pACDuet1; the protein contained no tag. All
217 constructs were under the control of an Isopropyl β-D-1-thiogalactopyranoside (IPTG)
218 inducible *lac* promoter. Plasmids pTRC-prfA and pET11a-prfB were introduced
219 separately into *E. coli* BL21(DE3)Gold cells (Agilent Technologies), already harbouring
220 pACDuet1-prmC, via chemical transformation. Protein production was induced with 1
221 mM IPTG in exponential phase cultures grown at 37 °C in LB medium. To allow full
222 methylation of the class I release factors, cells were further grown for 4 h. The bacteria
223 were lysed using a French press and purified using HisTrap nickel affinity
224 chromatography (GE Healthcare) per manufacturer's instructions. Purified proteins
225 were concentrated, dialyzed against 1 L of Polymix Buffer (pH 7.5) (Indrisiunaite et al.,
226 2015; Jelenc and Kurland, 1979), with one buffer exchange after 3 h, and stored at -
227 80 °C until use. Methylation of the release factors was verified by mass spectrometry
228 as described earlier (Heurgue-Hamard et al., 2002). For the *in vitro* assays, the
229 experiments were prepared and performed as described earlier (Korkmaz and Sanyal,
230 2017) with the following mRNAs 5′ GGG AAU UCG GGC CCU UGU UAA CAA UUA
231 AGG AGG UAU ACU <u>AUG</u> STOP CUG CAG $(A)_{21}$ 3′ (the start codon is underlined,
232 and STOP indicates the position of the stop codon).

233

		Cognate UGA		Non Cognate UGA		Accuracy Ratio
mRF1	kcat (s-1)	1.7	+/- 0.03	0.620	+/- 0.05	
	KM (nM)	5	/	164	+/- 3.0E-8	
	MM (s-1 nM-1)	0.340		0.004		
	Accuracy		**89.9**			3.63
RF1	kcat (s-1)	0.372	+/- 0.005	0.120	+/- 0.008	
	KM (nM)	5	/	40	+/- 1.8E-8	
	MM (s-1 nM-1)	0.074		0.003		
	Accuracy		**24.8**			
		UAA		UAG		
mRF2	kcat (s-1)	12*	+/- 1.1	1.490	+/- 0.1	
	KM (nM)	123*	+/- 2.9E-8	1045	+/- 3.42E-7	
	MM (s-1 nM-1)	0.097*		0.001		
	Accuracy		**68.4**			5.23
RF2	kcat (s-1)	0.75	+/- 0.03	0.560	+/- 0.03	
	KM (nM)	26	+/- 5.5E-9	254	+/- 3.5E-8	
	MM (s-1 nM-1)	0.029		0.002		
	Accuracy		**13.1**			

*Data from (Korkmaz and Sanyal, 2017).

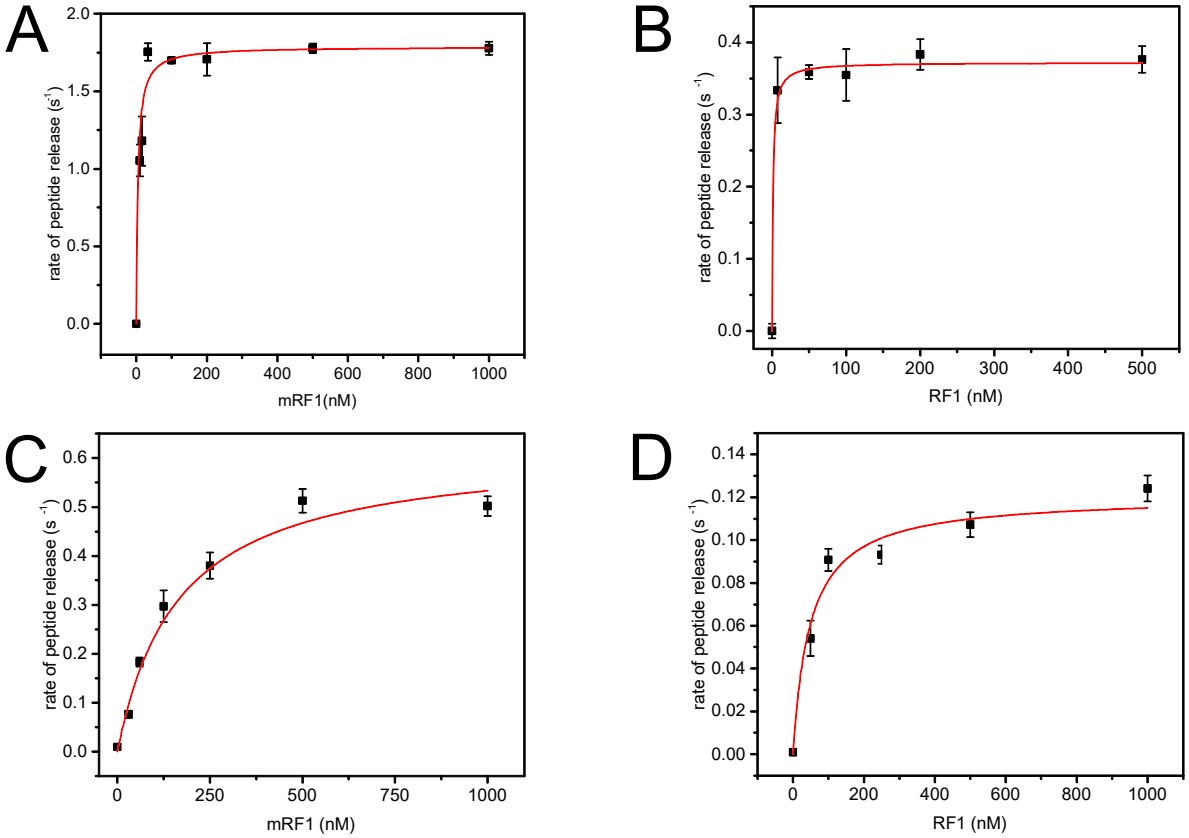

Figure 1 Michaelis-Menten graphs of class I release factors on cognate and non-cognate substrates. (A) mRF1 on UAA (B) RF1 on UAA (C) mRF1 on UGA (D) RF1 on UGA

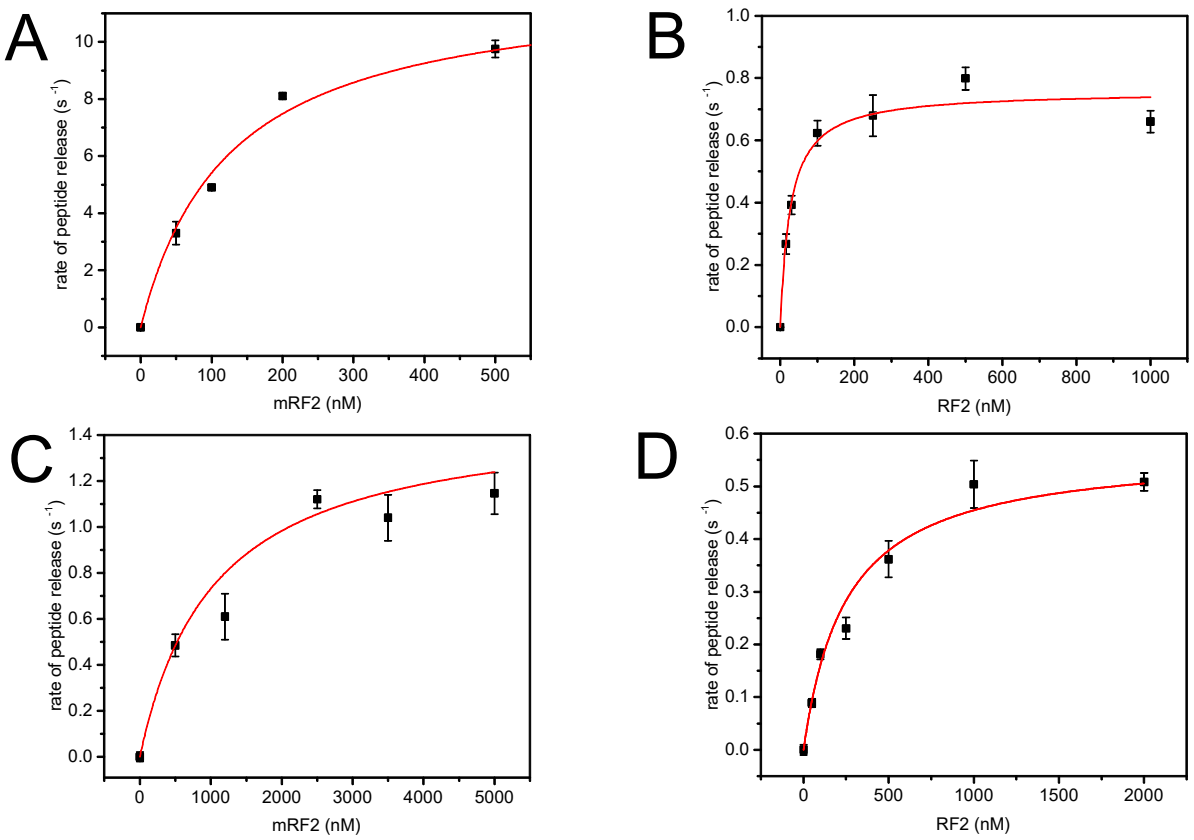

Figure 2 Michaelis-Menten graphs of class I release factors on cognate and non-cognate substrates.(A) mRF2 (Korkmaz and Sanyal, 2017) on UAA (B) RF2 on UAA (C) mRF2 on UAG (D) RF2 on UAG

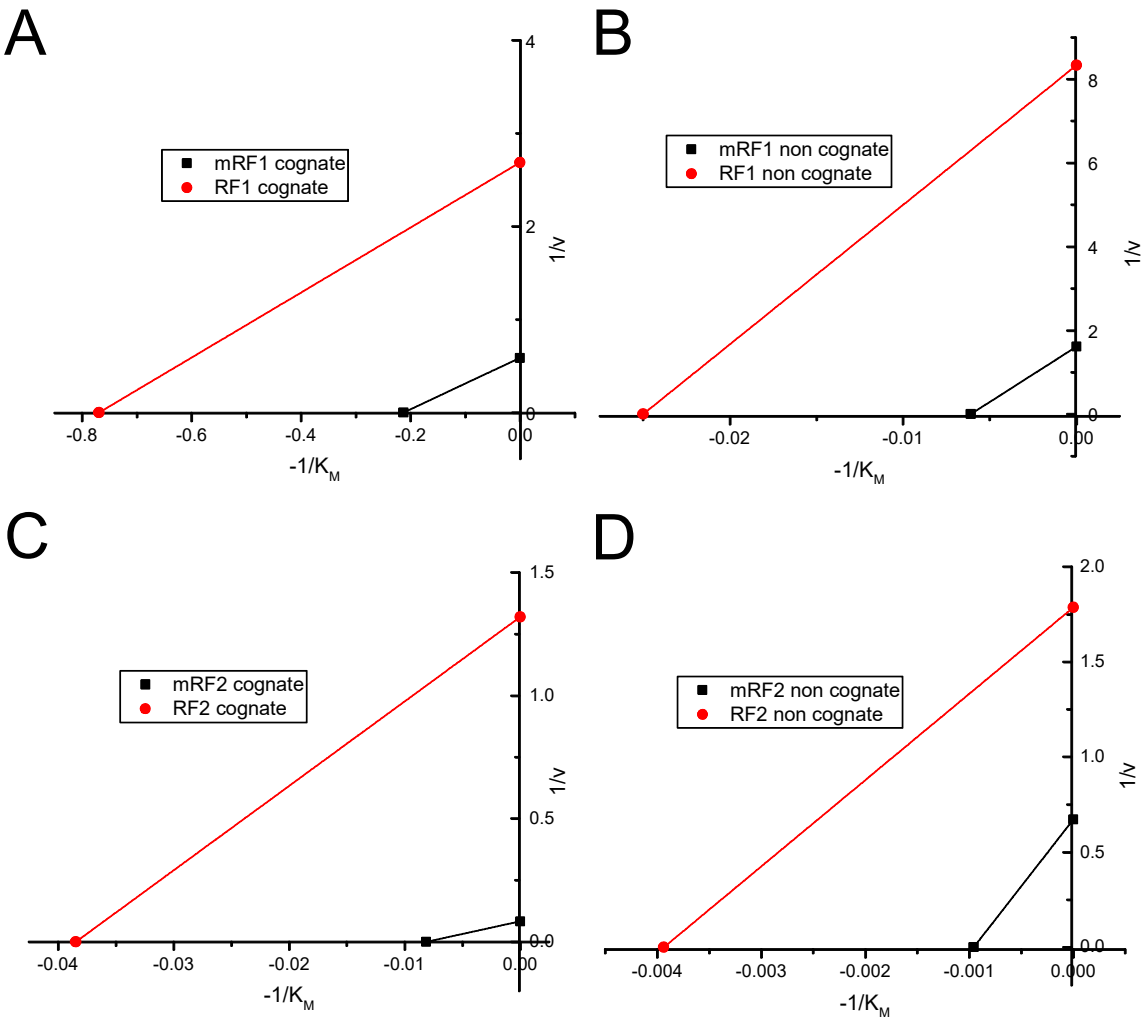

Figure 3 Lineweaver-Burk plots of kinetic data from Table 1. (A) Lineweaver-Burk plot comparison of methylated and non-methylated RF1 kinetic data on UAA. For illustration purposes, the K_M value for RF1 was evaluated as observed in Table 1. (B) Comparison of mRF1 and RF1 on UAG. (C) Comparison of mRF2 and RF2 on UAA. (D) Comparison of mRF2 and RF2 on UAG. All K_M values are given in nM.