1
2

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

3

4

5

6   Gürkan Korkmaz

7   Department of Cell and Molecular Biology, Uppsala University, Sweden

8

9

10   Correspondents to Gürkan Korkmaz via gurkankorkmaz@mail.com

11

12

13

14

15

16

17

18

19

20

23

24

25

26   **Abbreviations:** RF - release factor, RC - release complex

1

1 **ABSTRACT**

2 Ribosomal protein synthesis (translation) is a highly accurate process. Translation

3 termination, in particular, must be accurate to prevent truncated proteins. How this

4 accuracy is achieved is not fully understood in all its details. Using an *E. coli in vitro*

5 system, I explore novel mechanisms that contribute to the high accuracy of translation

6 termination. By comparing the Michaelis-Menten parameters of methylated and non-

7 methylated release factors on cognate and non-cognate codons. Post-translational

8 methylation of a strictly conserved GGQ motif in class I release factors increases the

9 accuracy of termination by up to 5-fold. This happens by increasing both the maximum

10 rate of peptide release (k_{cat}) and Michaelis-Menten constant (K_M). Further, I

11 demonstrate here that a non-methylated release factor acts like an uncompetitive

12 inhibitor of enzyme reactions. Overall, this study shows that the methylation of class I

13 release factors is a novel mechanism contributing to highly accurate translation

14 termination.

INTRODUCTION

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

Stop codon recognition is highly accurate *in vitro* (Freistroffer et al., 2000) and *in vivo* (Jørgensen et al., 1993), but our understanding of how this accuracy is achieved is still rudimentary. The accuracy of a reaction is defined as the ratio of the efficiency of a cognate to a non-cognate reaction (Freistroffer et al., 2000). The efficiency is determined by the ratio of the maximum rate of the reaction (k_{cat}) to the concentration at half k_{cat} (K_M). 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.

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

Highly conserved recognition motifs of RF1 and RF2 are located in the vicinity of the stop codons, but about 75Å distant from the catalytic center (Korostelev, 2011). These 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

4

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).

69   **RESULTS & DISCUSSION**

70   In this study, I investigated the methylation of the GGQ glutamine as an additional

71   factor that might enhance the accuracy of translation termination *in vitro*. The accuracy

72   of a reaction is defined as the ratio of the efficiency (k_{cat} / K_M) of a cognate to a non-

73   cognate reaction (Freistroffer et al., 2000). When the ratio of cognate and non-cognate

74   reaction is one, accuracy is non-existent, meaning no discrimination between the

75   cognate and non-cognate substrate are observable. I systematically determined the

76   k_{cat} and K_M parameters for release factors on various purified release complexes

77   (Korkmaz and Sanyal, 2017) containing cognate and non-cognate codons in A site

78   and compared the resulting accuracy with the reactions involving methylated (mRF1

79   or mRF2) and non-methylated (RF1 or RF2) release factors (Table 1 and Figure 2 and

80   3). UAA was used as the cognate codon in all experiments, while UAG and UGA were

81   used as the non-cognate substrates for RF1 and RF2 respectively since these codons

82   allow discrimination similar to non-cognate sense codons (Freistroffer et al., 2000).

83   The determined cognate k_{cat} parameters were in agreement with previous data for non-

84   methylated release factors (Bouakaz et al., 2006; Freistroffer et al., 2000) and also

85   with recent studies that used methylated release factors (Indrisiunaite et al., 2015;

86   Pierson et al., 2016). I observed a 4.5-fold increase in the maximum rate of peptide

87   release for mRF1 on its cognate UAA codon and a 5-fold increase in the maximum

88   rate of tritium-labeled fMet (^{3H}fMet) release on the non-cognate codon UGA. However,

89   it should be noted that for the cognate reactions, the K_M values for reactions with either

90   mRF1 or RF1 were below the detection limit of 5 nM. Thus, the real difference in K_M

91   might be much larger than what could be measured in these experiments. In Table 1

92   the estimated K_M parameters are listed. The K_M value form RF1 on UGA decreased

93   by 3-fold, from 164 nM to 48 nM, when methylation was missing. These resulted in an

6

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

7

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 1). 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.

CONFLICT OF INTEREST

No conflict of interest is stated.

REFERENCES

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

197 Seit-Nebi, A., Frolova, L., Justesen, J., and Kisselev, L. (2001). Class-1 translation
198 termination factors: invariant GGQ minidomain is essential for release activity and
199 ribosome binding but not for stop codon recognition. Nucleic Acids Res *29*, 3982–
200 3987.

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

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

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

208

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

12

|		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**			

234

235 *Data from (Korkmaz and Sanyal, 2017).

236
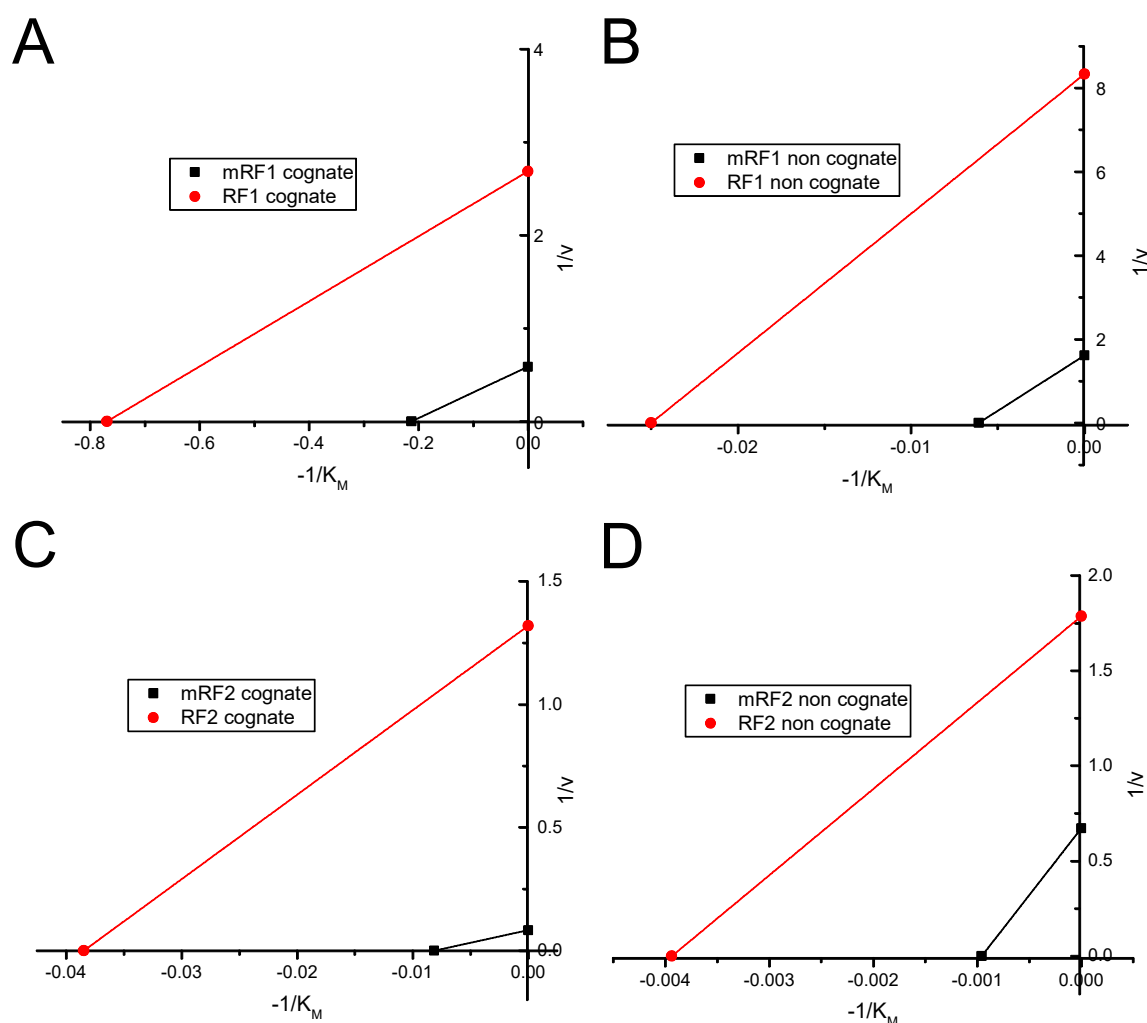
Figure 1 Lineweaver-Burk plots of kinetic data from Table 1. (A) Lineweaver-Burk plot comparison of methylated and non-methylated RF1 kinetic data on UAA. For illustration purposes, the K_M value for RF1 was evaluated as observed in Table 1. (B) Comparison of mRF1 and RF1 on UAG. (C) Comparison of mRF2 and RF2 on UAA. (D) Comparison of mRF2 and RF2 on UAG. All K_M values are given in nM.
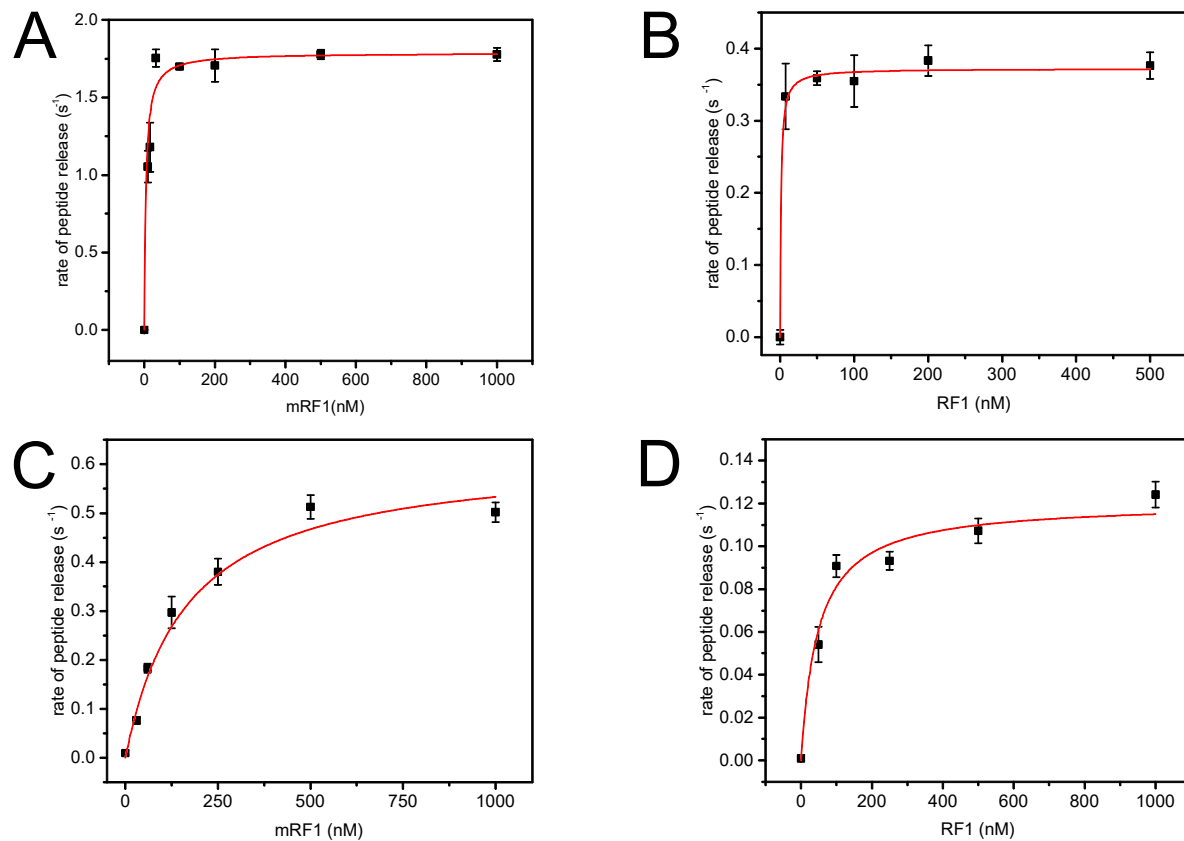
Figure 2 Michaelis-Menten graphs of class I release factors on cognate and non-cognate substrates. (A) mRF1 on UAA (B) RF1 on UAA (C) mRF1 on UGA (D) RF1 on UGA
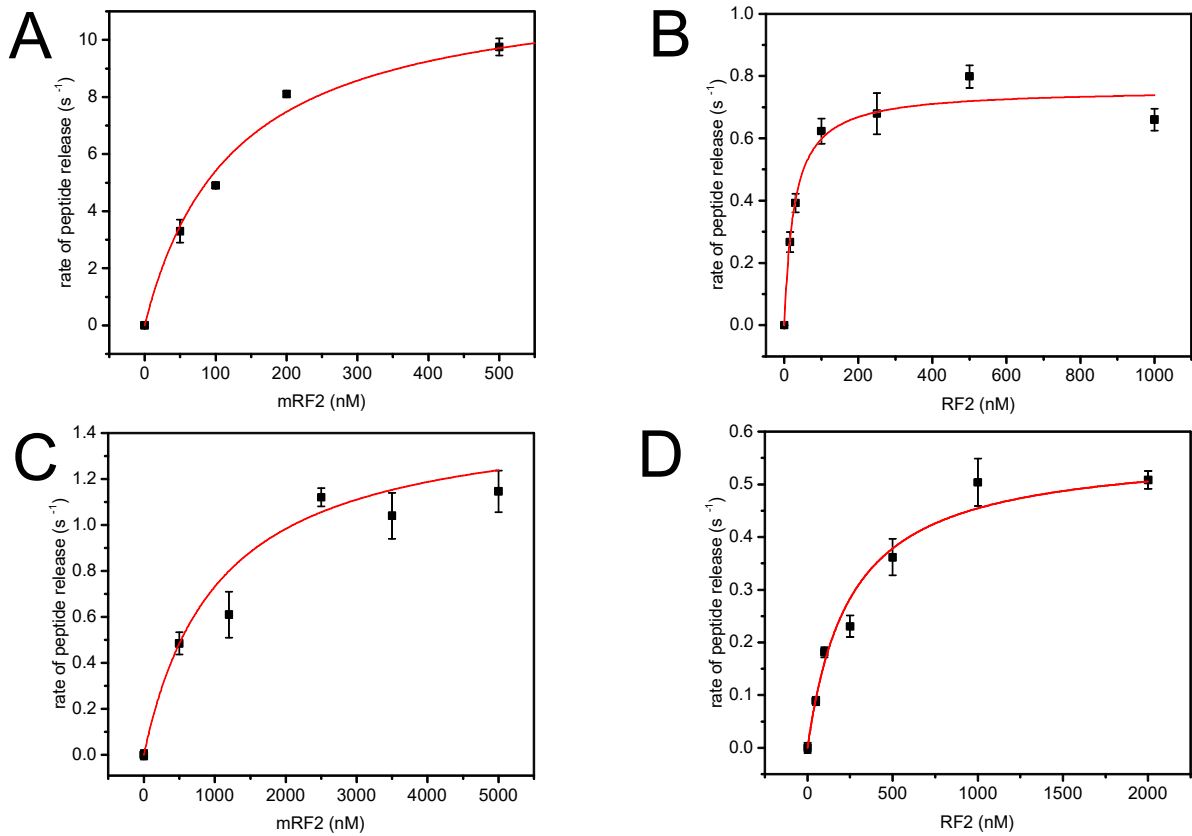
Figure 3 Michaelis-Menten graphs of class I release factors on cognate and non-cognate substrates.(A) mRF2 (Korkmaz and Sanyal, 2017) on UAA (B) RF2 on UAA (C) mRF2 on UAG (D) RF2 on UAG