Exploration of optimal conditions for the incorporation of noncanonical amino acids by amber suppression using the PURE system

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<Abstract>

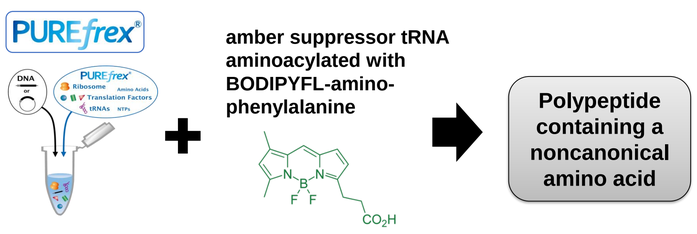
PURE*frex*® is a reconstituted cell-free protein synthesis system based on the PURE system, composed solely of factors essential for protein synthesis in *Escherichia coli* (*E. coli*). Due to its highly adjustable reaction composition, PURE*frex* is well-suited for the amber suppression method, in which non-canonical amino acid (ncAA) is incorporated at an amber stop codon (UAG) using a suppressor tRNA charged with the ncAA. However, detailed optimization of the reaction conditions for amber suppression using the PURE system has not been thoroughly investigated.

In this study, we explored the optimal conditions for amber suppression using PURE*frex*. As a model protein, we used a construct in which S-tag (KETAAAKFERSQHMDS) was fused to the N-terminus of *E. coli* dihydrofolate reductase (DHFR). We synthesized the fusion protein in the presence of an amber suppressor tRNA with BODIPY FL-aminophenylalanine (BFLAF) from the template DNA containing an amber codon within S-tag. The suppression efficiency was evaluated by measuring the fluorescence of BODIPY in the synthesized protein.

First, we examined the optimal position of an amber codon within S-tag. As a result, the suppression efficiency was high when an amber codon was introduced at positions T7 and F12. Next, using these constructs, we optimized the concentration of some components and BFLAF-charged suppressor tRNA in the reaction mixture. Unexpectedly, we found that the addition of release factor 1 (RF1), which recognizes an amber codon, improved the suppression efficiency. In addition, we found that the codon immediately preceding an amber codon significantly affected the suppression efficiency and the yield of full-length protein.

We believe these results provide valuable insights for amber suppression using PURE*frex*.

Incorporation of noncanonical amino acids by amber suppression using PURE*frex*®



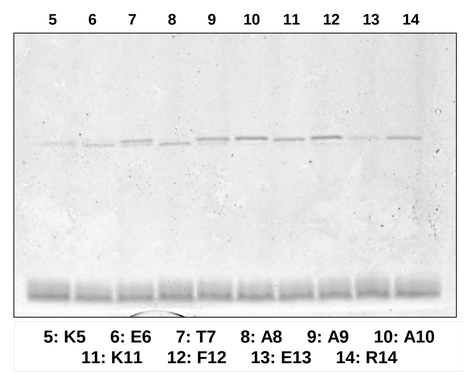
Purpose of this study

Exploration of the optimal conditions for incorporation of a noncanonical amino acid into the N-terminal region by amber suppression

1. Optimal position of amber codon for incorporation of a noncanonical amino acid

Model protein: Stag-DHFR-FLAG

MNKL | KETAAAKFERSQHMDS | DHFR | FLAG



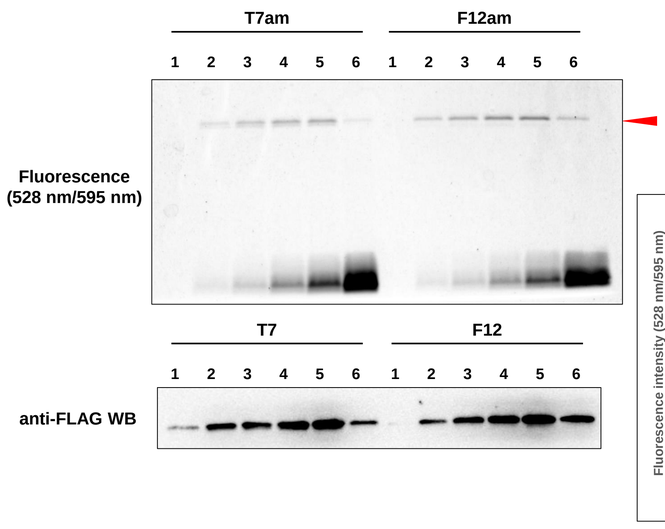
ver. 1 (ΔRF1)
+ 0.5 μg/μL amber suppressor tRNA aminoacylated with BODIPYFL-amino-phenylalanine (BFLAF-tRNAcua)
1 ng/μL Stag(am)-DHFR-FLAG
reaction at 37 ºC for 60 min

The amount of the product containing BODIPY was higher at positions T7, A10 and F12, than at other positions. However, the efficiency of the incorporation of BFLAF was low.

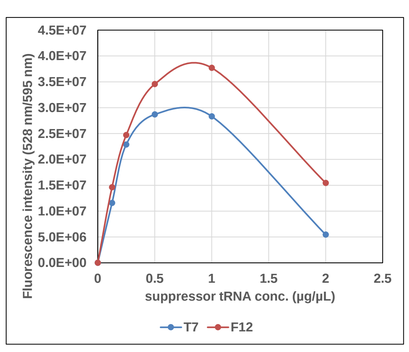
2. Optimization of the concentration of three factors in the reagent for amber suppression

2-1. suppressor tRNA

ver. 1 (ΔRF1)
+ 0-2 μg/μL BFLAF-tRNAcua
1 ng/μL Stag(T7am or F12am)-DHFR-FLAG
reaction at 37 ºC for 60 min



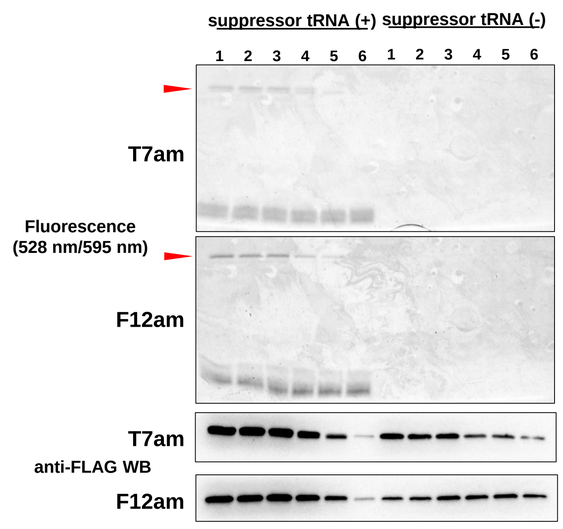
suppressor tRNA conc.
1: (-)
2: 0.125 μg/μL (x1/4)
3: 0.25 μg/μL (x1/2)
4: 0.5 μg/μL (x1)
5: 1 μg/μL (x2)
6: 2 μg/μL (x4)



The product containing BFLAF exhibited high expression levels when 0.5 or 1 μg/μL of suppressor tRNA was applied. However, the productivity was decreased when 2 μg/μL suppressor tRNA was used.

2-2. RF1

ver. 1 (ΔRF1)
+ 0/0.25 μg/μL BFLAF-tRNAcua
+ 0-10 μM RF1
1 ng/μL Stag(T7am or F12am)-DHFR-FLAG
reaction at 37 ºC for 60 min



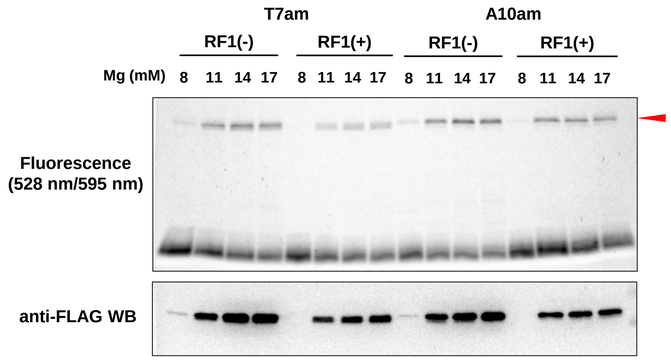
RF1 conc.
1: (-)
2: 1 nM
3: 10 nM
4: 100 nM
5: 1 μM
6: 10 μM

The suppression efficiency was comparable to that observed in the absence of RF1, even at concentrations below 100 nM. Additionally, full-length products were synthesized without using suppressor tRNA.

The presence of RF1 is recommended to prevent the synthesis of full-length products incorporating near-cognate tRNA and to achieve precise amber suppression.

2-3. Magnesium

ver. 1 (ΔRF1/ΔCTP/ΔUTP/ΔT7RNAP)
+ 0.25 μg/μL BFLAF-tRNAcua
+ 0/0.1 μM RF1
Mg: 8-17 mM
0.2 μM Stag(T7am or A10)-DHFR-FLAG mRNA
reaction at 37 ºC for 60 min



The product containing BFLAF was synthesized with high efficiency at 14 mM magnesium or higher.

sup. tRNA: 0.5-1 μg/μL
RF1: 0-100 nM
Magnesium: > 14 mM

3. Optimal codon immediately before amber codon

In *E. coli* S30-extract system, it has been shown that the codon immediately preceding amber codon affects the efficiency of amber suppression using BFLAF.

Construction of DNA in which the codon immediately preceding amber codon was substituted

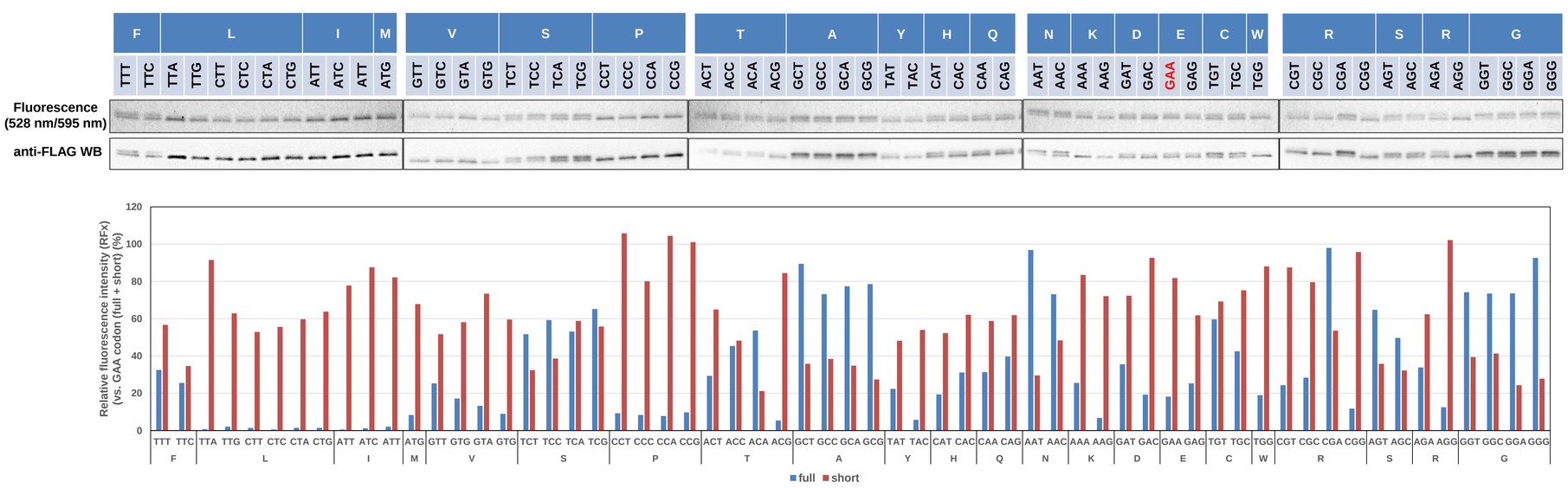
Stag (X6T7am)-DHFR-FLAG

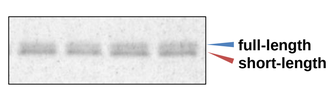
MNKL | KX*AAAKFERSQHMDS | DHFR | FLAG

X=F (ttt)
F (ttc)
L (tta)
L (ttg)
…….
} 60 types of X6T7am constructs

*=tag (amber codon)

PUREfrex ver. 1 (ΔRF1)
+ 0.25 μg/μL BFLAF-tRNAcua
+ 100 nM RF1
1 ng/μL Stag(X6T7am)-DHFR-FLAG
reaction at 37 ºC for 60 min

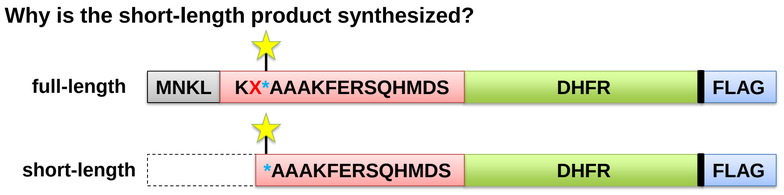




$$RF_X\,(\%) = \frac{FL_X}{FL_{GAA\text{-}full} + FL_{GAA\text{-}short}} \times 100$$

RF_X : Relative fluorescence intensity of the X codon
FL_X : Fluorescence intensity of the X codon
$FL_{GAA\text{-}full}$: Fluorescence intensity of the full-length GAA codon
$FL_{GAA\text{-}short}$: Fluorescence intensity of the short-length GAA codon

Why is the short-length product synthesized?



It is hypothesized that a product with an N-terminal amber codon is synthesized.

The codon immediately upstream of amber codon significantly affected the synthesis by amber suppression using PUREfrex.

Good codon: Ala (GCT/ GCC/ GCA/ GCG)
Gly (GGT/ GGC/ GGA/ GGG)
Arg (CGA)
Asn (AAT)

4. Conclusion

- **A noncanonical amino acid was incorporated by amber suppression even in the presence of RF1 (or it could be better with RF1).**
- **The position of amber codon and a codon immediately before amber codon significantly affected the suppression efficiency.**

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