Towards scalable cell-free synthesis of non-native amino acid containing proteins

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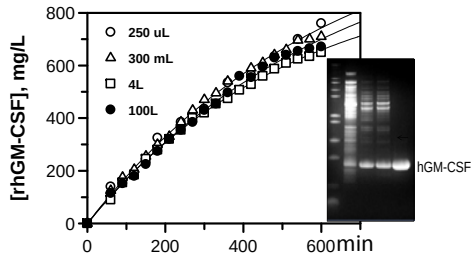
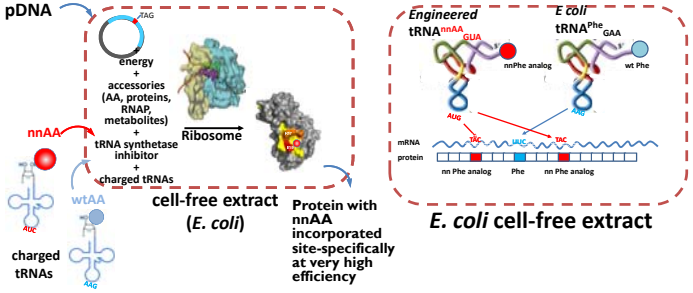
Abstract: Sutro Biopharma has developed an open cell-free expression system (OCFS) for high-yield production of proteins to the 100 L scale. We have extended this technology for the incorporation non-native amino acids (nnAAs) into proteins with ≥99% efficiency. Described is a method for tRNA aminoacylation with nnAAs at large scale. These nnAA-charged tRNAs were used in cell-free reactions to site-specifically incorporate nnAAs. This approach obviates the need for an orthogonal tRNA-synthetase pair and allows for greater control over the site-specific incorporation of nnAAs. By utilizing this method our technology can lead to the creation of novel protein and peptide libraries that are not limited to the 20 naturally-occurring amino acids.

Linear scalable high yield production of fully soluble, bioactive Granulocyte-Macrophage Colony-Stimulating Factor (**hGM-CSF**) to 100L in a cell free reaction.



- Recovery at high yield and in high purity at scale
- Correct mass
- The 2 disulfide bonds are correctly formed

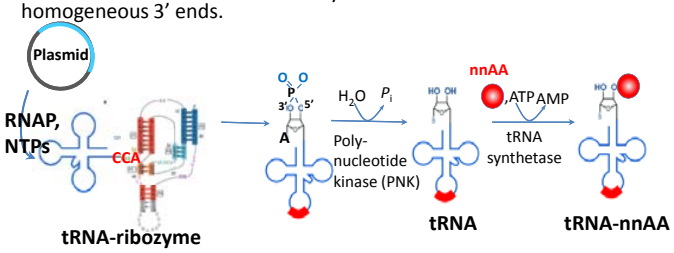
Dual-charging cell free reaction scheme: charged tRNAs are added exogenously to direct the site-specific incorporation of non-native amino acids. Endogenous charging is inhibited. Preventing tRNA recharging allows for complete control over nnAA incorporation.



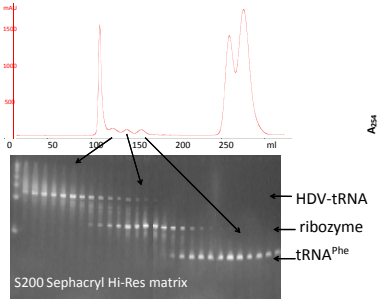
Scalable production of charged tRNAs is required for both AA-tRNAs and nnAA-tRNA that are added to the cell free reaction. We have made 100s of mgs of $tRNA^{Phe}$'s using processes that will allow for the cost effective production of tRNA at the gram to kg scale with the goal of producing proteins containing nnAA's at the kg scale

A high-copy-number plasmid encoding tRNA and HDV ribozyme is used for *in vitro* transcription. Ribozyme autocatalytic cleavage ensures homogeneous 3' ends.

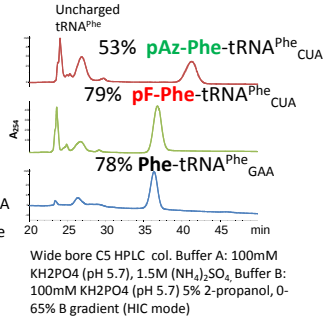
After transcription and purification by SEC, tRNAs are treated with polynucleotide kinase to remove the 3'-5' cyclic phosphate resulting from HDV cleavage and then charged with desired wild-type or nnAA using engineered tRNA syntheases.



tRNAs can be purified by size

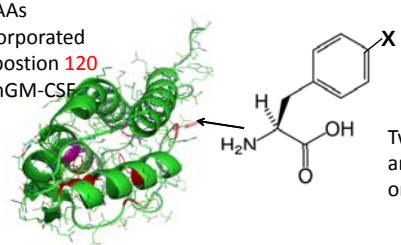


Rapid HPLC assay for tRNA charging



Wide bore C5 HPLC col. Buffer A: 100mM KH_2PO_4 (pH 5.7), 1.5M $(NH_4)_2SO_4$ Buffer B: 100mM KH_2PO_4 (pH 5.7) 5% 2-propanol, 0-65% B gradient (HIC mode)

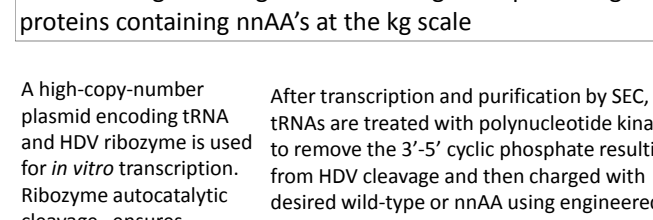
nnAAs incorporated at postion 120 of hGM-CSF



X = *p*-fluoro *p*-azido

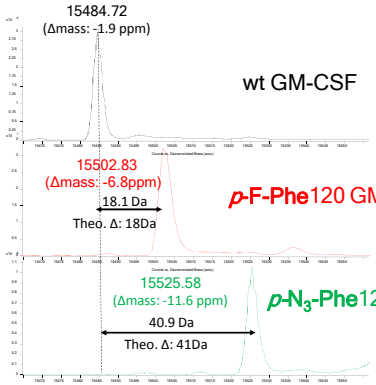
Two phenylalanine analogs were charged on *E coli* $tRNA^{Phe}_{CUA}$

MAPARSPSPSTQPWEHVNAIQEARRLLNLSRDTAAEMNETVEVISE
M**F**DLQEPTCLQTRLEL**Y**KQGLRGSLTKLKGPLTMMASH**Y**KQHCPPT
PETSCATQIIT**F**ES**F**KENLKD**F**LLVIP**F**DCWEPVQE



Unoptimized nnAA incorporation reactions contained 80 µM Phe-$tRNA^{Phe}_{GAA}$ and 30 uM nnAA-$tRNA^{Phe}_{CUA}$. Control WT GM-CSF reactions were without PheRS inhibitor or added tRNAs.

Mass spectrometry analysis confirms highly efficient (≥99%) nnAA incorporation of both analogs. No wild-type phenylalanine could be detected at position 120 within the limit of detection.



Summary

- We have extended the utility of our scalable cell-free protein synthesis technology for the expression of proteins with nnAAs.
- nnAAs are incorporated site-specifically and highly-efficiently, making it possible to engineer novel protein structures and functions.
- Efforts at moving cell-free synthesis technologies to cGMP manufacturing are underway.