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Technical Note

Cell-Free Translation Quantification via a Fluorescent Minihelix

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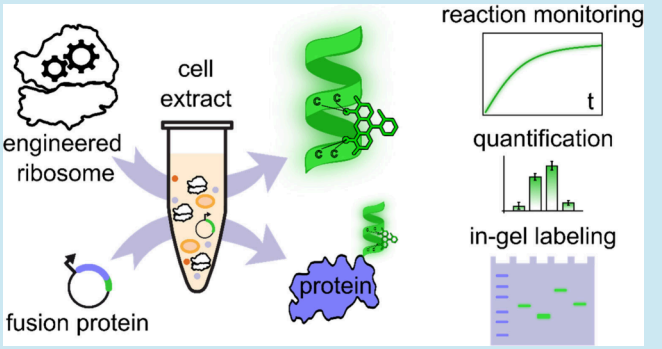
ABSTRACT: Cell-free gene expression systems are used in numerous applications, including medicine making, diagnostics, and educational kits. Accurate quantification of nonfluorescent proteins in these systems remains a challenge. To address this challenge, we report the adaptation and use of an optimized tetra-cysteine minihelix both as a fusion protein and as a standalone reporter with the FlAsH dye. The fluorescent reporter helix is short enough to be encoded on a primer pair to tag any protein of interest via PCR. Both the tagged protein and the standalone reporter can be detected quantitatively in real time or at the end of cell-free expression reactions with standard 96/384-well plate readers, an RT-qPCR system, or gel electrophoresis without the need for staining. The fluorescent signal is stable and correlates linearly with the protein concentration, enabling product quantification. We modified the reporter to study cell-free expression dynamics and engineered ribosome activity. We anticipate that the fluorescent minihelix reporter will facilitate efforts in engineering *in vitro* transcription and translation systems.



KEYWORDS: *fluorescent reporter, protein quantification, cell-free gene expression, high-throughput, synthetic biology, in vitro transcription and translation*

INTRODUCTION

Cell-free gene expression (CFE) systems have matured as a powerful technology platform for synthetic biology.[1−3] CFE systems find utility in accelerating biological design[4−8] as well as in biomanufacturing,[9−14] portable diagnostics,[15−18] and educational kits,[19−23] among others. To support such development, accurate and reliable quantification of protein synthesis is important. Yet, protein quantification remains a bottleneck for the field.

Protein quantification in CFE systems typically relies on several methods. Common methods include radioactive amino acid incorporation,[24,25] FluoroTect labeling,[26] and fusions to GFP or split-GFP.[27−29] Unfortunately, such methods are not accessible to all laboratories (e.g., radioactive counting) or result in bulky modifications to the protein being measured (e.g., GFP fusions).

Here, to address these limitations, we report a simple, accessible assay for real-time monitoring of protein translation in cell-free systems based on the profluorescent dye 4,5-bis(1,3,2-dithiarsolan-2-yl)fluorescein (FlAsH-EDT$_2$). FlAsH-EDT$_2$ binds reversibly to a bipartite tetra-cysteine structure within two turns of an α-helix[30] to emit fluorescence in the green spectrum (absorption: 508 nm, emission: 528−538 nm). The α-helix was optimized to allow for stronger binding of the FlAsH dye[31] and elevate quantum yield.[32] The fluorescent protein tag/dye pair has been characterized *in vivo* for imaging and quantification[30,31,33−35] and is available as an in-cell detection kit (LumioGreen, Invitrogen), but it has not, to our knowledge, been previously reported for use with CFE systems. To adapt the assay to cell-free systems, we expressed the optimized tetra-cysteine helix both as a fusion protein and as a standalone reporter in an *in vitro* assay with the FlAsH dye.

RESULTS

FlAsH-Based Assay Development for Protein Quantification in Cell-Free Systems. We set out to adapt the tetra-cysteine motif/FlAsH dye pair to a CFE environment (Figure 1A). To do this, we first tested the motif as a standalone reporter transcribed from a T7 promoter-driven plasmid and translated into the short fluorescence-emitting tetra-cysteine helix[32] in the CFE system. We found that the fluorescence signal after subtracting the background was linearly proportional to the amount of reporter present (Figure 1B), so it can be used for quantification purposes and scales with reaction volume. The presence of the FlAsH-EDT$_2$ dye does not appear to interfere with transcription or synthesis (Figure S1A).

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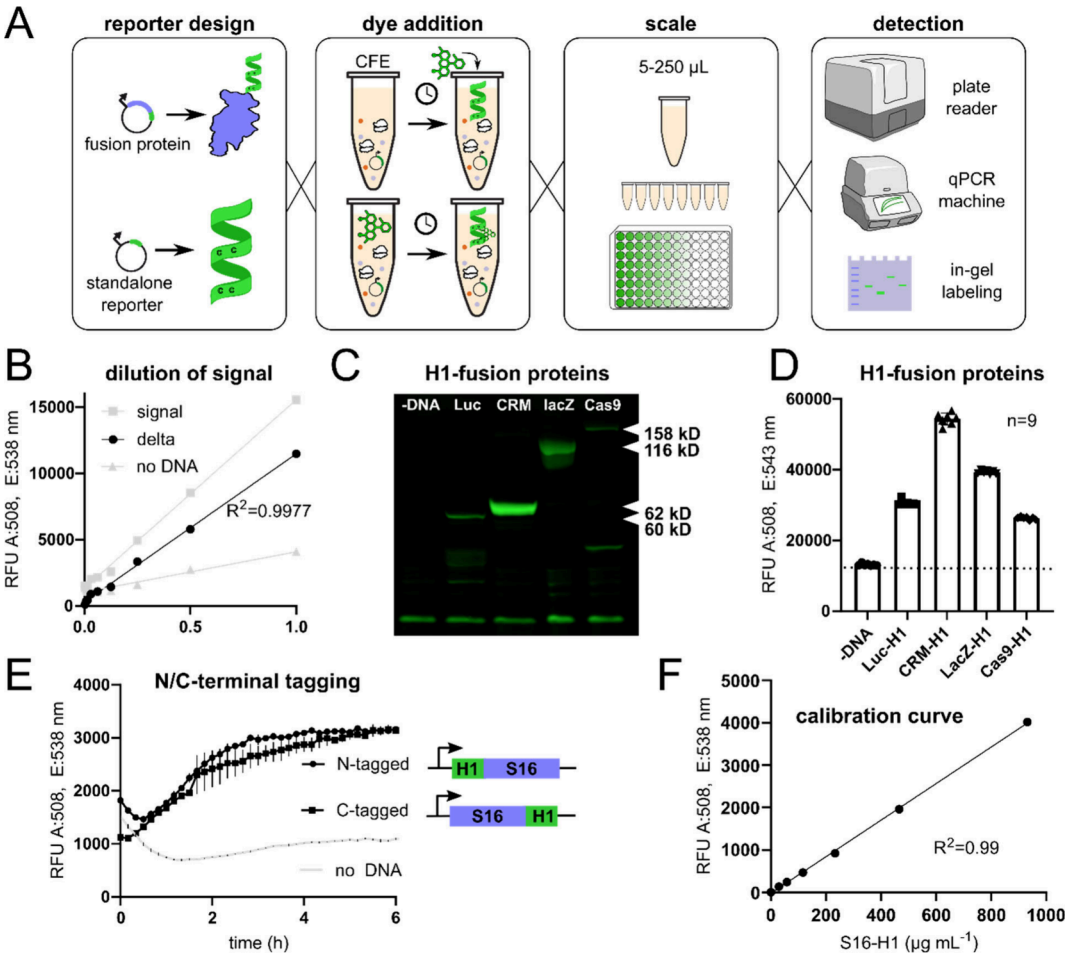

Figure 1. Fluorescent minihelix dye reporter for the detection and quantification of cell-free gene expression. (A) We present an assay that is flexible in reporter design, timing of the dye addition, scale and vessel format, and detection, all of which can be combined to tailor the assay to a multitude of applications. (B) CFE reactions expressing either H1 reporter helix (signal) or no DNA were serially diluted in PBS and their signal measured. The background-subtracted signal (delta) was fitted linearly and goes through the 0/0 point. (C, D) Fusion proteins for tracking protein expression in CFE. CFE reactions expressing either firefly luciferase, CRM197-4xDQNAT, LacZ, or SpCas9 proteins fused C-terminally to reporter helix H1 were either (C) separated on NuPAGE 4−12%, Bis-Tris in MOPS buffer and imaged with Azure gel imager on the Cy3 setting (see Figure S2B for uncropped gel image) or (D) measured on a plate reader after 10 h at 30 °C. No DNA negative control represents the autofluorescence of the dye interacting with cysteine-rich proteins present in the extract. Bar graph depicts mean and standard deviation of $n = 9$ reactions. (E) Proteins can be tagged both N- or C-terminally: We tagged reporter H1 either up or downstream of ribosomal protein S16 from *T. thermophilus* and detected the CFE produced fusion proteins in a plate reader in real time. Error bars represent the standard deviation of $n = 3$ experiments. (F) Fluorescence output is linearly correlated with protein yield: S16-H1 was expressed in the presence of ^{14}C-leucine and FlAsH-EDT$_2$, and the reaction split into two equal serial dilutions, one of which was measured on a plate reader to detect RFU, and the other was measured in a scintillation counter to calculate the exact concentration of S16-H1 protein in μg/mL.

However, the dye can interact with other cysteine-rich proteins found in cell extracts or translation systems[36] and produce a background signal in the absence of the desired tetra-cysteine helix reporter. We next tested whether thiol moieties such as ethane-1,2-dithiol (EDT), β-metcaptoethanol (β-ME), or dithiothreitol (DTT) can quench this undesired background fluorescence. We found the final concentration of 15 μM FlAsH with 2 mM β-ME to be the optimal signal to background for real-time observation of CFE reactions (Figure S1B).

Due to the covalent bonds formed, the helix dye product and resulting fluorescent signal are stable. We stored plates of completed reactions at room temperature on the benchtop for multiple weeks, measuring every few days (Figure S1C), and found that the fluorescent signal deteriorated at a rate of less than 3% per day. This stability means that when live reaction monitoring is not required, researchers with limited plate reader capacity may set up many plates in parallel and quantify them at the end point.

We also tested different reporter architectures, either standalone or fused to other functional tags sFLAG or StrepII tag, and found that our initial H1 minihelix construct yielded the highest signal with and without function tag additions (Figure S1D), likely due to a more stable minihelix under CFE conditions. While the H1 sequence (FLNCCPGCCMEP) performed the best in our *in vitro* assay, the sequence can be altered to be shorter and contain other residues or functional sequences on both the N- or C-terminus of the helix, so long as the central tetra-cysteine motif (CCPGCC) and helical structure is maintained.

We found this assay to be highly flexible, and it can be varied in several ways (Figure 1A): (i) the reporter minihelix can be expressed either as a standalone reporter of translation activity or as a tag to track the expression of the protein of interest. (ii)

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The FlAsH-EDT$_2$ dye can be added either to a completed CFE reaction (e.g., if reactions are split for other analysis) or at the start of the reaction to obtain kinetic measurements without taking aliquots. (iii) Just like CFE, the assay scales well, and reactions can be set up in microtubes, PCR tubes, or 96/384-well plates. (iv) The fluorescent signal can be detected via a plate reader, qPCR machine, or in-gel with imaging. The flexible reaction and detection setup allows the fluorescent minihelix reporter to be used in many possible applications and in instrument-limited settings.

Tetra-Cysteine Helix Fusion Proteins for Real-Time Protein Translation Monitoring. Tetra-cysteine helix labeling offers a simple route to tag and monitor proteins produced via cell-free gene expression. We first tested this approach by creating fusion proteins of various sizes. We C-terminally tagged the proteins firefly luciferase (bioluminescent enzyme, Uniprot P08659), CRM197-4xDQNAT (carrier protein used in conjugate vaccines),[10] LacZ (β-galactosidase, used in metabolic engineering and biosensors, Uniprot P00722), and SpCas9 (used in genetic engineering applications).[37] All fusion proteins were expressed and could be visualized in denaturing gel electrophoresis (Figure 1C), where the fluorescent helix did not add significant mass, making each protein migrate in the expected kDa ranges. Since the dye is already covalently bound to the reporter helix, the gel can be imaged without staining after the run, even without removing it from the glass plates. Background binding can be reduced by the addition of 25 mM β-ME before loading the gel (Figure S2A), and the specific signal allows for visualization of fusion protein products that would be indistinguishable from total lysate proteins in Coomassie staining (Figure S2B). The same reactions quantified on a plate reader matched the expression levels of each protein (Figure 1D).

We next tested the effect of the positioning of the fluorescent helix by C- and N-terminally tagging proteins of interest. We found that positioning did not appear to affect protein expression or the fluorescence yield in our example of *Thermus thermophilus* rspP (ribosomal protein S16, Uniprot Q5SJH3) (Figures 1E and S2C). However, results may differ for proteins with highly structured ends: positioning the tag N-terminally on luciferase approximately halved the luminescence yield, while the C-terminal tag had no significant effect on yield or activity (Figure S3). The tetra-cysteine minihelix behaves similarly to other tags, where previous findings showed that short tags may lower yield or activity of some proteins,[38−40] especially if added on the N-terminus. To date, no general rule exists to predict how a given tag will affect the expression and activity of a given protein, including this fluorescent minihelix.[39] Our real-time monitoring suggests that reaction kinetics change based on tag position, protein length, and complexity of the Open Reading Frame (ORF) (compare Figures 1E and 2B). This means tetra-cysteine helix tagging in CFE presents a general tool for tracking and comparing translation dynamics of different ORFs. Since net fluorescence linearly correlates with protein concentration (Figure 1B), we found that the assay output can be used qualitatively; however, we recommend creating a separate standard curve by radiolabeling a tetra-cysteine helix-tagged protein to allow conversion of relative fluorescence units (RFU) to protein concentration in μg/mL (Figure 1F). In our hands, the presence of FlAsH and β-ME in the CFE reaction did not interfere with protein yield (Figure S1A) or enzymatic activity (luminescence produced by luciferase, Figure S3), although we

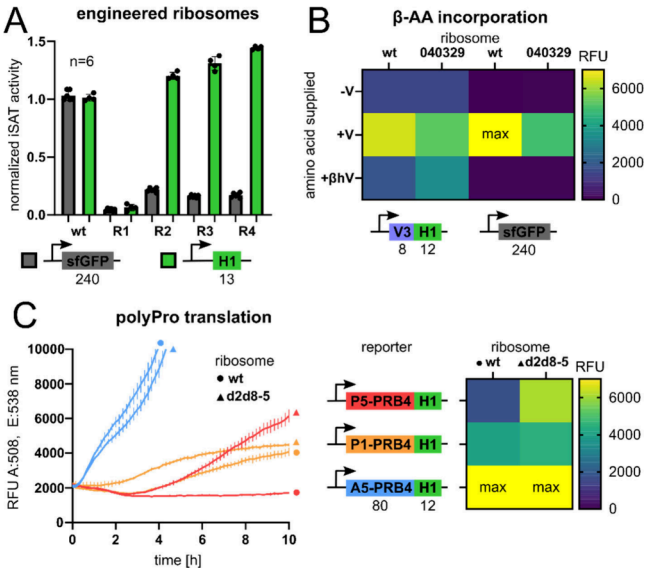

Figure 2. Fluorescent minihelices as standalone reporters for ribosome engineering. (A) iSAT activity of ribosome constructs with engineered rRNA expressing either sfGFP or the H1 minireporter. Wild type (wt) reactions are expressing a full-length 23S rRNA, while engineered ribosomes R1−R4 are expressing 23S rRNA variants with a 25 nt deletion in domain II, replaced with different linkers (all rRNA sequences are detailed in Table S2). sfGFP and H1 were detected on their respective optimal channels. The background was subtracted from all samples before normalizing to wt activity. Bar graphs depict mean and standard deviation of n = 6 experiments. (B) Expression of valine-coupled H1 reporter or sfGFP in the absence or presence of α-valine or β-homovaline by either wt ribosomes or mutant 040329. Average RFU of n = 3 reactions without background subtracted. (C) iSAT expression of polyproline-rich protein PRB4 followed by H1 for detection by either ● wild type ribosomes or ▲ d2d8-5 mutant, evolved specifically for increased polyproline expression.[45] Average RFU of n = 3 reactions without background subtracted. Error bars represent the standard deviation. Heat map on the right depicts end point quantifications of polyproline reporters by either ribosome type.

cannot guarantee that this is the case for every protein of interest. If protein folding or activity are of concern, researchers should omit FlAsH/β-ME from the CFE reaction or activity assay and then quantify the protein of interest afterward by adding the dye to the spent cell-free reaction.

A Peptide-Based Reporter for Ribosome Engineering. With the fluorescent minihelix system adapted to CFE, we next set out to apply the reporter for monitoring translation in engineered ribosomes. Engineering ribosomes could lead to the synthesis of new-to-nature biopolymers.[41−44] We hypothesize that short peptides (e.g., short open reading frame with peptide tags) would be more accommodating for translationally challenged engineered ribosomes over large α-amino acid-based reporter proteins, such as sfGFP (240 amino acids) or luciferase (562 amino acids), which are standard in CFE systems.

Here, we assessed whether the FlAsH-based assay could serve as a high-throughput, real-time alternative for measuring the translation output. To do this, we first adapted the assay to an integrated synthesis, assembly, and translation (iSAT) system, in which ribosomes are synthesized and assembled from an rDNA plasmid template and then functionally tested for protein biosynthesis.[46−48] iSAT can be used for testing engineered ribosomes.[43,49,50] We demonstrated that the tetra-

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ACS Synth. Biol. 2024, 13, 2253−2259

cysteine helix can be optimized to work with synthetic ribosomes produced in iSAT (Figure S4). For instance, while the ribosomes we engineered to have altered 23S rRNA sequences (Figure 2A, R1−R4) were largely inactive in producing sfGFP, several ribosome variants could instead translate the 13 amino acid H1 minihelix reporter. Thus, our assay captures a broader range of activity through allowing comparison of engineered ribosomes that would have been deemed nonfunctional in a standard assay synthesizing a larger protein.

We next showed that ribosomes engineered for noncanonical amino acid incorporation can more easily translate this short ORF compared to sfGFP. We designed a short peptide containing H1 on the C-terminal end of the sequence (M)LVMVLVM, which did not hinder fluorescent readout due to cushioning V with pro-helical residues. We then used iSAT to incorporate β-h-valine, which is charged onto $tRNA^{Val}$ by the endogenous valyl-tRNA synthetase,[51] in this reporter peptide with wild type (wt) and engineered ribosomes (Figure 2B). While wild type ribosomes can produce some fluorescence in the presence of β-h-Val, ribosome mutant 040329, evolved to better incorporate β-amino acids,[52,53] is able to produce a stronger signal. This assay leverages the minihelix reporter to produce a high-throughput compatible signal despite global replacement of amino acids with noncanonical alternatives, circumventing the need for orthogonal translation machinery, as used previously.[52,54,55] The same is not possible with GFP (Figure 2B), in which neither the wild type nor the 040329 ribosome produces a signal in the presence of β-h-Val.

Our assay can be combined with challenging ORFs to track ribosome evolution toward specific sequence specialization. For instance, stretches of polyproline are challenging for native ribosomes to translate due to proline's backbone clashing with rRNA residues, which induces stalling and requires resolution by elongation factor P.[56] To measure polyproline stalling, we constructed a reporter containing five prolines, a segment of polyproline-rich protein PRB4, and reporter helix H1 (Figure 2C, P5). Other reporters contained the same segments but only one proline (P1) prior to PBR4; and a proline-free control with the identical sequence with every proline replaced with alanine (A5), in both the presequence and PBR4. The expression of all constructs was tracked via the fluorescence of C-terminal minihelix H1. Indeed, wild type ribosomes could only efficiently express the A5 reporter, while engineered ribosome d2d8-5 was able to synthesize polyproline stretches in both the P5 and P1 constructs. Ribosome mutant d2d8-5 is the product of a previous directed evolution campaign toward specialization in polyproline translation.[45] While this assay is also possible with GFP instead of the tetra-cysteine helix, it is likely that proline-friendly ribosome variants would be less processive in translating proline-poor sequences and would thus be disadvantaged synthesizing GFP compared to wild type ribosomes. The fluorescent minihelix provides a high-throughput signal without placing a significant translational burden on engineered ribosomes.

In summary, this work illustrates the use of a fluorescent minihelix based on the FlAsH-interacting tetra-cysteine tag to quantify protein production in cell-free gene expression systems as well as engineered ribosomes. The assay is easy to use, quantitative, scalable, high-throughput compatible, and sensitive. It costs less than FluoroTect or radiolabeling, can adapt to various readout methods, and produces a long-lasting signal. Disadvantages include some background fluorescence by unspecific binding, which can be mitigated by the addition of thiol compounds. In some proteins, the addition of the fluorescent minihelix tag may alter expression, folding, and enzyme activity. This phenomenon has been documented for other tags and may limit the proteins that can be quantified and studied via this assay. Looking forward, we anticipate that this assay will help democratize and standardize protein quantification in cell-free systems and allow novel ways to assess the fitness of engineered ribosomes.

METHODS

Dye Preparation. FlAsH-EDT_2 (Cayman Chem, no. 20704) was suspended in DMSO to a concentration of 500 μM, stored at −20 °C. A fresh 15× concentrated mixture of FlAsH-EDT_2 and β-mercaptoethanol (Sigma-Aldrich) was prepared in PBS buffer and incubated for 15 min at room temperature (RT), prior to being added to the CFE reaction. Final concentrations of the dye were optimized for each use case and are detailed below.

Reporter Plasmids. Fetch reporters were constructed on a pJL1 plasmid (T7 promoter, KanR) compatible with the protein synthesis assays. All reporters were constructed by site-directed mutagenesis via PCR sing Q5 High-Fidelity DNA Polymerase (NEB) to create overlaps forming the Fetch sequence followed by Gibson assembly and transformation into DH10β cells. The Fetch variants (H1: FLNCCPGCCMEP and H2: HRWCCPGCCKTF) were either cloned directly between start and stop codon of the open reading frame of pJL1 or added either downstream of sFLAG tag or upstream of StrepII tag. H1 was also cloned C-terminally to pJL1-CRM197-4xDQNAT, -LacZ, and -SpCas9, as well as V3, polyPro reporters P5, P1, and A5 (full sequences in Supplementary Table S1). All reporters were confirmed by Sanger sequencing and deposited to Addgene (see Supporting Information for the Addgene no. of each reporter construct). Plasmid pJL1-sfGFP (Addgene #102634) was used as a control for CFE activity. All plasmids were isolated from cells via miniprep (Zymo kit).

CFE Reactions. Cell-free protein synthesis reactions were prepared by using BL21 Star (DE3) cell lysate. Lysate preparation and optimized CFE reaction compositions were based on previous works.[1,57−59] The 15 μL CFE reactions included 0.1 μg/μL T7 pol, 2 mM DTT, 13 ng/μL reporter plasmid, and the optimized final concentration of 15 μM FlAsH-EDT_2 and 2 mM β-ME unless otherwise specified (in the case of real-time monitoring). CFE reactions were incubated at 30 °C for 10 h while monitored, as described below. Alternatively, dye-free reactions can be set up and incubated for 30 °C for 20 h to completion, then mixed 1:1 with 10 μM FlAsH-EDT_2 and 10 mM β-ME in PBS and incubated at room temperature for 1 h before quantification.

Fluorescence Detection. Detection of tetra-cysteine-FlAsH complex was ideally performed in a Biotek Synergy H1 plate reader by defining a read channel specific to FlAsH (absorption, 508 nm; emission, 538 nm) or, alternatively, in a Biorad CFX96 Touch Real-Time PCR Detection System by using the "all channels" setting and later selecting the HEX channel (Ex, 515−535 nm; Em, 560−580 nm) to evaluate. A negative control reaction containing no reporter DNA was always run to determine background fluorescence, specifically dye binding to cell lysate. A reaction expressing sfGFP can serve as a positive control for extract activity but should be

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monitored on a separate channel (absorption, 485 nm; emission, 528 nm). End point values were quantified by averaging the last five data points of a given reaction.

Radiolabeling. Total CFE yields of *T. thermophilus* S16 protein were quantified by incorporation of ^{14}C-leucine (PerkinElmer), as previously described.[24,25] ^{14}C-leucine was included in CFE reactions to reach a final concentration of 10 μM in triplicate 15 μL reactions and incubated overnight at 30 °C, as previously described.[10,60,61] Then, 5 μL of each reaction was mixed with an equivalent volume of 0.5N KOH and incubated for 20 min at 37 °C to deacylated tRNA. Next, 5 μL of each reaction mixture was spotted onto two separate 96-well filter mats (PerkinElmer 1450-421) and dried under a heat lamp. One of the mats was washed three times for 15 min in 5% TCA at 4 °C to precipitate protein and washed once in 100% EtOH before being fully dried under a heat lamp. Radioactivity was measured by a liquid scintillation counter (PerkinElmer MicroBeta) compared with the unwashed filter mat. Protein concentration was calculated from the measured scintillation count per reaction volume by total number of Leu per S16 protein (7L), ^{14}C-Leu specific activity (300 mCi/mmol), and molar mass of S16 (10.4 kDa).

Gel Separation and Imaging. The 5 μL amount of CFE reactions already containing FlAsH dye was brought to 15 μL with PBS, mixed with 5 μL of 4× NuPAGE LDS Sample Buffer (Thermo Fisher), and heated to 70 °C for 10 min. Then, 25 mM β-mercaptoethanol was added to the samples before loading to further reduce background. Proteins were separated on appropriate polyacrylamide gels, e.g., NuPAGE 4−12%, Bis-Tris in MOPS running buffer for larger proteins, and 20% Bis-Tris in Tris-glycine buffer for products below 40 kDa. Gels were run at 100 V with BenchMark Fluorescent Protein Standard (Thermo) and imaged immediately after completion using either Cytiva Amersham ImageQuant 800 or Azure Biosystems 200 imager on channel Cy3 (535 nm/UV). Coomassie staining of total proteins was performed using InstantBlue (Novus Bio) for gel documentation purposes.

iSAT Reactions. iSAT reactions allow for testing ribosomal variants encoded in a plasmid in a cell-free translation system, as previously described,[46−48] and iSAT reactions were set up as detailed therein, with each reaction containing 3.1 ng/μL pJL1-reporter plasmid as specified in each figure and 14 ng/μL pT7rrn plasmid encoding the rRNA variants (full 23S rDNA sequences in Suppl. Table S2), as well as 7.5 μM FlAsH-EDT$_2$ and 2 mM β-ME. Standard 15 μL reactions furthermore contained 8 mM magnesium glutamate, 10 mM ammonium glutamate, 130 mM potassium glutamate, 0.85 mM each of GTP, UTP, and CTP, 1.2 mM ATP, 34 μg/mL folinic acid, 0.171 mg/mL *Escherichia coli* tRNA, 0.33 mM NAD, 0.27 mM CoA, 4 mM oxalic acid, 1 mM putrescine, 1.5 mM spermidine, 57 mM HEPES, 2 mM 20 amino acids, 37 mM PEP, ∼300 nM total protein of the 70S ribosome (TP70), 60 μg/mL T7 RNA polymerase, 0.50 μL of PEG-8000 40% (Sigma-Aldrich), and 5 μL of S150 extract. Reactions were incubated at 37 °C for 10 h, and fluorescence was detected as described above.

Luciferase Assay. CFE reactions were set up as described above, expressing either the Luc-H1 reporter (N- or C-terminal) or untagged Luc. Reactions either included both 5 μM FlAsH-EDT$_2$ and 2 mM β-ME or just PBS instead. Reactions were incubated at 30 °C. The completed reactions were supplemented with 1 vol of PBS containing nothing else or the appropriate amount of dye to result in a final concentration of 5 μM FlAsH-EDT$_2$ and 2 mM β-ME; then, fluorescence was detected as described above. Next, 4 μL of these samples (equal to a 2 μL CFE reaction) was mixed into 30 μL of OneGlo reagent (Promega) in a chilled, white 96-well plate, and luminescence was read on a Biotek Synergy 2 plate reader at 26 °C.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.4c00266.

Figures documenting optimization of fluorescent mini-helix assay for *in vitro* detection and quantification of cell-free expression products, extended gel images, exprewssion of reporter variants, and tagged luciferase assays; tables of reporter protein and nucleotide sequences (with Addgene ID numbers) and rRNA sequences (PDF)

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Author Contributions

J.A.W. designed, performed, and analyzed the experiments. A.K. and M.C.J. directed the study and interpreted the data. J.A.W., A.K., and M.C.J. wrote and edited the manuscript.

Notes

The authors declare the following competing financial interest(s): M.C.J. has a financial interest in Resilience, Gauntlet Bio, Synolo Therapeutics, and Stemloop, Inc. The interests of M.C.J. are reviewed and managed by Northwestern University and Stanford University in accordance with their conflict-of-interest policies. All other authors declare no conflicts of interest.

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REFERENCES

(1) Silverman, A. D.; Karim, A. S.; Jewett, M. C. Cell-free gene expression: an expanded repertoire of applications. *Nature Reviews Genetics 2019 21:3* **2020**, *21*, 151−170.

(2) Garenne, D.; Haines, M. C.; Romantseva, E. F.; Freemont, P.; Strychalski, E. A.; Noireaux, V. Cell-free gene expression. *Nat. Rev. Methods Primers* **2021**, *1*, 49.

(3) Carlson, E. D.; Gan, R.; Hodgman, C. E.; Jewett, M. C. Cell-free protein synthesis: applications come of age. *Biotechnol Adv.* **2012**, *30*, 1185−1194.

(4) Pandi, A.; Adam, D.; Zare, A.; Trinh, V. T.; Schaefer, S. L.; Burt, M.; Klabunde, B.; Bobkova, E.; Kushwaha, M.; Foroughijabbari, Y.; Braun, P.; Spahn, C.; Preußer, C.; Pogge von Strandmann, E.; Bode, H. B.; von Buttlar, H.; Bertrams, W.; Jung, A. L.; Abendroth, F.; Schmeck, B.; Hummer, G.; Vázquez, O.; Erb, T. J. Cell-free biosynthesis combined with deep learning accelerates de novo-development of antimicrobial peptides. *Nat. Commun.* **2023**, *14*, 7197.

(5) Hunt, A. C.; Vögeli, B.; Hassan, A. O.; Guerrero, L.; Kightlinger, W.; Yoesep, D. J.; Krüger, A.; DeWinter, M.; Diamond, M. S.; Karim, A. S.; Jewett, M. C. A rapid cell-free expression and screening platform for antibody discovery. *Nat. Commun.* **2023**, *14*, 3897.

(6) Hunt, A. C.; Case, J. B.; Park, Y. J.; Cao, L.; Wu, K.; Walls, A. C.; Liu, Z.; Bowen, J. E.; Yeh, H. W.; Saini, S.; Helms, L.; Zhao, Y. T.; Hsiang, T. Y.; Starr, T. N.; Goreshnik, I.; Kozodoy, L.; Carter, L.; Ravichandran, R.; Green, L. B.; Matochko, W. L.; Thomson, C. A.; Vögeli, B.; Krüger, A.; VanBlargan, L. A.; Chen, R. E.; Ying, B.; Bailey, A. L.; Kafai, N. M.; Boyken, S. E.; Ljubetic, A.; Edman, N.; Ueda, G.; Chow, C. M.; Johnson, M.; Addetia, A.; Navarro, M. J.; Panpradist, N.; Gale, M.; Freedman, B. S.; Bloom, J. D.; Ruohola-Baker, H.; Whelan, S. P. J.; Stewart, L.; Diamond, M. S.; Veesler, D.; Jewett, M. C.; Baker, D. Multivalent designed proteins neutralize SARS-CoV-2 variants of concern and confer protection against infection in mice. *Sci. Transl Med.* **2022**, *14*, eabn1252.

(7) Liew, F. E.; Nogle, R.; Abdalla, T.; Rasor, B. J.; Canter, C.; Jensen, R. O.; Wang, L.; Strutz, J.; Chirania, P.; De Tissera, S.; Mueller, A. P.; Ruan, Z.; Gao, A.; Tran, L.; Engle, N. L.; Bromley, J. C.; Daniell, J.; Conrado, R.; Tschaplinski, T. J.; Giannone, R. J.; Hettich, R. L.; Karim, A. S.; Simpson, S. D.; Brown, S. D.; Leang, C.; Jewett, M. C.; Köpke, M. Carbon-negative production of acetone and isopropanol by gas fermentation at industrial pilot scale. *Nature Biotechnology 2022 40:3* **2022**, *40*, 335−344.

(8) Karim, A. S.; Dudley, Q. M.; Juminaga, A.; Yuan, Y.; Crowe, S. A.; Heggestad, J. T.; Garg, S.; Abdalla, T.; Grubbe, W. S.; Rasor, B. J.; Coar, D. N.; Torculas, M.; Krein, M.; Liew, F.; Quattlebaum, A.; Jensen, R. O.; Stuart, J. A.; Simpson, S. D.; Köpke, M.; Jewett, M. C. In vitro prototyping and rapid optimization of biosynthetic enzymes for cell design. *Nat. Chem. Biol.* **2020**, *16*, 912−919.

(9) Des Soye, B. J.; Gerbasi, V. R.; Thomas, P. M.; Kelleher, N. L.; Jewett, M. C. A Highly Productive, One-Pot Cell-Free Protein Synthesis Platform Based on Genomically Recoded Escherichia coli. *Cell Chem. Biol.* **2019**, *26*, 1743−1754.

(10) Stark, J. C.; Jaroentomeechai, T.; Moeller, T. D.; Hershewe, J. M.; Warfel, K. F.; Moricz, B. S.; Martini, A. M.; Dubner, R. S.; Hsu, K. J.; Stevenson, T. C.; Jones, B. D.; DeLisa, M. P.; Jewett, M. C. On-demand biomanufacturing of protective conjugate vaccines. *Sci. Adv.* **2021**, *7*, eabe9444.

(11) Warfel, K. F.; Williams, A.; Wong, D. A.; Sobol, S. E.; Desai, P.; Li, J.; Chang, Y. F.; DeLisa, M. P.; Karim, A. S.; Jewett, M. C. A Low-Cost, Thermostable, Cell-Free Protein Synthesis Platform for On-Demand Production of Conjugate Vaccines. *ACS Synth. Biol.* **2023**, *12*, 95−107.

(12) Martin, R. W.; Majewska, N. I.; Chen, C. X.; Albanetti, T. E.; Jimenez, R. B. C.; Schmelzer, A. E.; Jewett, M. C.; Roy, V. Development of a CHO-Based Cell-Free Platform for Synthesis of Active Monoclonal Antibodies. *ACS Synth. Biol.* **2017**, *6*, 1370−1379.

(13) Yin, G.; Garces, E. D.; Yang, J.; Zhang, J.; Tran, C.; Steiner, A. R.; Roos, C.; Bajad, S.; Hudak, S.; Penta, K.; Zawada, J.; Pollitt, S.; Murray, C. J. Aglycosylated antibodies and antibody fragments produced in a scalable in vitro transcription-translation system. *MAbs* **2012**, *4*, 217−225.

(14) Groff, D.; Carlos, N. A.; Chen, R.; Hanson, J. A.; Liang, S.; Armstrong, S.; Li, X.; Zhou, S.; Steiner, A.; Hallam, T. J.; Yin, G. Development of an E. coli strain for cell-free ADC manufacturing. *Biotechnol. Bioeng.* **2022**, *119*, 162.

(15) Jung, J. K.; Alam, K. K.; Verosloff, M. S.; Capdevila, D. A.; Desmau, M.; Clauer, P. R.; Lee, J. W.; Nguyen, P. Q.; Pastén, P. A.; Matiasek, S. J.; Gaillard, J. F.; Giedroc, D. P.; Collins, J. J.; Lucks, J. B. Cell-free biosensors for rapid detection of water contaminants. *Nature Biotechnology 2020 38:12* **2020**, *38*, 1451−1459.

(16) Thavarajah, W.; Silverman, A. D.; Verosloff, M. S.; Kelley-Loughnane, N.; Jewett, M. C.; Lucks, J. B. Point-of-Use Detection of Environmental Fluoride via a Cell-Free Riboswitch-Based Biosensor. *ACS Synth. Biol.* **2020**, *9*, 10−18.

(17) Pardee, K.; Green, A. A.; Ferrante, T.; Cameron, D. E.; Daleykeyser, A.; Yin, P.; Collins, J. J. Paper-based synthetic gene networks. *Cell* **2014**, *159*, 940−954.

(18) Pardee, K.; Green, A. A.; Takahashi, M. K.; Braff, D.; Lambert, G.; Lee, J. W.; Ferrante, T.; Ma, D.; Donghia, N.; Fan, M.; Daringer, N. M.; Bosch, I.; Dudley, D. M.; O'Connor, D. H.; Gehrke, L.; Collins, J. J. Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components. *Cell* **2016**, *165*, 1255−1266.

(19) Jung, J. K.; Rasor, B. J.; Rybnicky, G. A.; Silverman, A. D.; Standeven, J.; Kuhn, R.; Granito, T.; Ekas, H. M.; Wang, B. M.; Karim, A. S.; Lucks, J. B.; Jewett, M. C. At-Home, Cell-Free Synthetic Biology Education Modules for Transcriptional Regulation and Environmental Water Quality Monitoring. *ACS Synth. Biol.* **2023**, *12*, 2909−2921.

(20) Stark, J. C.; Huang, A.; Nguyen, P. Q.; Dubner, R. S.; Hsu, K. J.; Ferrante, T. C.; Anderson, M.; Kanapskyte, A.; Mucha, Q.; Packett, J. S.; Patel, P.; Patel, R.; Qaq, D.; Zondor, T.; Burke, J.; Martinez, T.; Miller-Berry, A.; Puppala, A.; Reichert, K.; Schmid, M.; Brand, L.; Hill, L. R.; Chellaswamy, J. F.; Faheem, N.; Fetherling, S.; Gong, E.; Gonzalzles, E. M.; Granito, T.; Koritsaris, J.; Nguyen, B.; Ottman, S.; Palffy, C.; Patel, A.; Skweres, S.; Slaton, A.; Woods, T. R.; Donghia, N.; Pardee, K.; Collins, J. J.; Jewett, M. C. BioBits™ Bright: A fluorescent synthetic biology education kit. *Sci. Adv.* **2018**, *4*, eaat5107.

(21) Huang, A.; Nguyen, P. Q.; Stark, J. C.; Takahashi, M. K.; Donghia, N.; Ferrante, T.; Dy, A. J.; Hsu, K. J.; Dubner, R. S.; Pardee, K.; Jewett, M. C.; Collins, J. J. Biobits™ explorer: A modular synthetic biology education kit. *Sci. Adv.* **2018**, *4*, eaat5105.

(22) Stark, J. C.; Huang, A.; Hsu, K. J.; Dubner, R. S.; Forbrook, J.; Marshalla, S.; Rodriguez, F.; Washington, M.; Rybnicky, G. A.; Nguyen, P. Q.; Hasselbacher, B.; Jabri, R.; Kamran, R.; Koralewski, V.; Wightkin, W.; Martinez, T.; Jewett, M. C. BioBits Health: Classroom Activities Exploring Engineering, Biology, and Human Health with Fluorescent Readouts. *ACS Synth. Biol.* **2019**, *8*, 1001−1009.

(23) Williams, L. C.; Gregorio, N. E.; So, B.; Kao, W. Y.; Kiste, A. L.; Patel, P. A.; Watts, K. R.; Oza, J. P. The Genetic Code Kit: An Open-Source Cell-Free Platform for Biochemical and Biotechnology Education. *Front. Bioeng. Biotechnol.* **2020**, *8*, 941.

(24) Kim, D. M.; Kigawa, T.; Choi, C. Y.; Yokoyama, S. A Highly Efficient Cell-Free Protein Synthesis System from Escherichia coli. *Eur. J. Biochem.* **1996**, *239*, 881−886.

(25) Jewett, M. C.; Calhoun, K. A.; Voloshin, A.; Wuu, J. J.; Swartz, J. R. An integrated cell-free metabolic platform for protein production and synthetic biology. *Mol. Syst. Biol.* **2008**, *4*, 220.

(26) Kobs, G.; Hurst, R.; Betz, N.; Godat, B. FluoroTect™ Green Lys in vitro Translation Labeling System. In *Promega Notes*; Promega Corporation, 2001; Vol. *77*, p 23−27.

https://doi.org/10.1021/acssynbio.4c00266
ACS Synth. Biol. 2024, 13, 2253−2259

(27) Kahn, T. W.; Beachy, R. N.; Falk, M. M. Cell-free expression of a GFP fusion protein allows quantitation in vitro and in vivo. *Curr. Biol.* **1997**, *7*, R207−R208.
(28) Pham, T. D.; Poletti, C.; Tientcheu, T. M. N.; Cuccioloni, M.; Spurio, R.; Fabbretti, A.; Milon, P.; Giuliodori, A. M. FAST, a method based on split-GFP for the detection in solution of proteins synthesized in cell-free expression systems. *Sci. Rep.* **2024**, *14*, 8042.
(29) Karim, A. S.; Jewett, M. C. Cell-Free Synthetic Biology for Pathway Prototyping. In *Methods in Enzymology*, 1st ed.; Elsevier Inc., 2018; p 31−57.
(30) Griffin, B. A.; Adams, S. R.; Tsien, R. Y. Specific Covalent Labeling of Recombinant Protein Molecules Inside Live Cells. *Science (1979)* **1998**, *281*, 269−272.
(31) Adams, S. R.; Campbell, R. E.; Gross, L. A.; Martin, B. R.; Walkup, G. K.; Yao, Y.; Llopis, J.; Tsien, R. Y. New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: Synthesis and biological applications. *J. Am. Chem. Soc.* **2002**, *124*, 6063−6076.
(32) Martin, B. R.; Giepmans, B. N. G.; Adams, S. R.; Tsien, R. Y. Mammalian cell−based optimization of the biarsenical-binding tetracysteine motif for improved fluorescence and affinity. *Nature Biotechnology 2005 23:10* **2005**, *23*, 1308−1314.
(33) Hoffmann, C.; Gaietta, G.; Zürn, A.; Adams, S. R.; Terrillon, S.; Ellisman, M. H.; Tsien, R. Y.; Lohse, M. J. Fluorescent labeling of tetracysteine-tagged proteins in intact cells. *Nature Protocols 2010 5:10* **2010**, *5*, 1666−1677.
(34) Adams, S. R.; Tsien, R. Y. Preparation of the membrane-permeant biarsenicals FlAsH-EDT2 and ReAsH-EDT2 for fluorescent labeling of tetracysteine-tagged proteins. *Nature Protocols 2008 3:9* **2008**, *3*, 1527−1534.
(35) Albert Griffin, B.; Adams, S. R.; Jones, J.; Tsien, R. Y. Fluorescent labeling of recombinant proteins in living cells with FlAsH. *Methods Enzymol* **2000**, *327*, 565−578.
(36) Stroffekova, K.; Proenza, C.; Beam, K. G. The protein-labeling reagent FLASH-EDT2 binds not only to CCXXCC motifs but also non-specifically to endogenous cysteine-rich proteins. *Pflugers Arch* **2001**, *442*, 859−66.
(37) Escobar, M.; Li, J.; Patel, A.; Liu, S.; Xu, Q.; Hilton, I. B. Quantification of Genome Editing and Transcriptional Control Capabilities Reveals Hierarchies among Diverse CRISPR/Cas Systems in Human Cells. *ACS Synth. Biol.* **2022**, *11*, 3239−3250.
(38) Araújo, A. P. U.; Oliva, G.; Henrique-Silva, F.; Garratt, R. C.; Cáceres, O.; Beltramini, L. M. Influence of the Histidine Tail on the Structure and Activity of Recombinant Chlorocatechol 1,2-Dioxygenase. *Biochem. Biophys. Res. Commun.* **2000**, *272*, 480−484.
(39) Costa, S.; Almeida, A.; Castro, A.; Domingues, L. Fusion tags for protein solubility, purification and immunogenicity in Escherichia coli: the novel Fh8 system. *Front. Microbiol.* **2014**, *5*, 63.
(40) Dudley, Q. M.; Cai, Y.-M.; Kallam, K.; Debreyne, H.; Carrasco Lopez, J. A.; Patron, N. J. Biofoundry-assisted expression and characterization of plant proteins. *Synth Biol.* **2021**, *6*, ysab029.
(41) Kofman, C.; Lee, J.; Jewett, M. C. Engineering molecular translation systems. *Cell Syst* **2021**, *12*, 593−607.
(42) Kofman, C.; Willi, J. A.; Karim, A. S.; Jewett, M. C. Ribosome Pool Engineering Increases Protein Biosynthesis Yields. *ACS Cent Sci.* **2024**, *10* (4), 871.
(43) Krüger, A.; Watkins, A. M.; Wellington-Oguri, R.; Romano, J.; Kofman, C.; DeFoe, A.; Kim, Y.; Anderson-Lee, J.; Fisker, E.; Townley, J.; d'Aquino, A. E.; Das, R.; Jewett, M. C. Community science designed ribosomes with beneficial phenotypes. *Nat. Commun.* **2023**, *14*, 961.
(44) Orelle, C.; Carlson, E. D.; Szal, T.; Florin, T.; Jewett, M. C.; Mankin, A. S. Protein synthesis by ribosomes with tethered subunits. *Nature* **2015**, *524*, 119−124.
(45) Schmied, W. H.; Tnimov, Z.; Uttamapinant, C.; Rae, C. D.; Fried, S. D.; Chin, J. W. Controlling orthogonal ribosome subunit interactions enables evolution of new function. *Nature* **2018**, *564*, 444−448.
(46) Jewett, M. C.; Fritz, B. R.; Timmerman, L. E.; Church, G. M. In vitro integration of ribosomal RNA synthesis, ribosome assembly, and translation. *Mol. Syst. Biol.* **2013**, *9*, 678.
(47) Fritz, B. R.; Jewett, M. C. The impact of transcriptional tuning on in vitro integrated rRNA transcription and ribosome construction. *Nucleic Acids Res.* **2014**, *42*, 6774−85.
(48) Fritz, B. R.; Jamil, O. K.; Jewett, M. C. Implications of macromolecular crowding and reducing conditions for in vitro ribosome construction. *Nucleic Acids Res.* **2015**, *43*, 4774.
(49) Liu, Y.; Davis, R. G.; Thomas, P. M.; Kelleher, N. L.; Jewett, M. C. In vitro-Constructed Ribosomes Enable Multi-site Incorporation of Noncanonical Amino Acids into Proteins. *Biochemistry* **2021**, *60*, 161−169.
(50) Kofman, C.; Watkins, A. M.; Kim, D. S.; Willi, J. A.; Wooldredge, A. C.; Karim, A. S.; Das, R.; Jewett, M. C. Computationally-guided design and selection of high performing ribosomal active site mutants. *Nucleic Acids Res.* **2022**, *50*, 13143−13154.
(51) Hartman, M. C. T.; Josephson, K.; Szostak, J. W. Enzymatic aminoacylation of tRNA with unnatural amino acids. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 4356−4361.
(52) Maini, R.; Chowdhury, S. R.; Dedkova, L. M.; Roy, B.; Daskalova, S. M.; Paul, R.; Chen, S.; Hecht, S. M. Protein Synthesis with Ribosomes Selected for the Incorporation of *β*-Amino Acids. *Biochemistry* **2015**, *54*, 3694−3706.
(53) Lee, J.; Schwarz, K. J.; Kim, D. S.; Moore, J. S.; Jewett, M. C. Ribosome-mediated polymerization of long chain carbon and cyclic amino acids into peptides in vitro. *Nat. Commun.* **2020**, *11*, 4304.
(54) Dedkova, L. M.; Fahmi, N. E.; Paul, R.; del Rosario, M.; Zhang, L.; Chen, S.; Feder, G.; Hecht, S. M. *β*-Puromycin selection of modified ribosomes for in vitro incorporation of *β*-amino acids. *Biochemistry* **2012**, *51*, 401−15.
(55) Dedkova, L. M.; Hecht, S. M. Expanding the Scope of Protein Synthesis Using Modified Ribosomes. *J. Am. Chem. Soc.* **2019**, *141*, 6430−6447.
(56) Huter, P.; Arenz, S.; Bock, L. V.; Graf, M.; Frister, J. O.; Heuer, A.; Peil, L.; Starosta, A. L.; Wohlgemuth, I.; Peske, F.; Nováček, J.; Berninghausen, O.; Grubmüller, H.; Tenson, T.; Beckmann, R.; Rodnina, M. V.; Vaiana, A. C.; Wilson, D. N. Structural Basis for Polyproline-Mediated Ribosome Stalling and Rescue by the Translation Elongation Factor EF-P. *Mol. Cell* **2017**, *68*, 515−527.
(57) Jewett, M. C.; Swartz, J. R. Mimicking the Escherichia coli cytoplasmic environment activates long-lived and efficient cell-free protein synthesis. *Biotechnol. Bioeng.* **2004**, *86*, 19−26.
(58) Jewett, M. C.; Calhoun, K. A.; Voloshin, A.; Wuu, J. J.; Swartz, J. R. An integrated cell-free metabolic platform for protein production and synthetic biology. *Mol. Syst. Biol.* **2008**, *4*, 220.
(59) Kwon, Y.-C.; Jewett, M. C. High-throughput preparation methods of crude extract for robust cell-free protein synthesis. *Sci. Rep.* **2015**, *5*, 8663.
(60) Kightlinger, W.; Duncker, K. E.; Ramesh, A.; Thames, A. H.; Natarajan, A.; Stark, J. C.; Yang, A.; Lin, L.; Mrksich, M.; DeLisa, M. P.; Jewett, M. C. A cell-free biosynthesis platform for modular construction of protein glycosylation pathways. *Nat. Commun.* **2019**, *10*, 5404.
(61) Stark, J. C.; Jaroentomeechai, T.; Warfel, K. F.; Hershewe, J. M.; DeLisa, M. P.; Jewett, M. C. Rapid biosynthesis of glycoprotein therapeutics and vaccines from freeze-dried bacterial cell lysates. *Nature Protocols 2023 18:7* **2023**, *18*, 2374−2398.

https://doi.org/10.1021/acssynbio.4c00266
ACS Synth. Biol. 2024, 13, 2253−2259