OPTIMIZING PROTEIN EXPRESSION IN THE ONE-POT PURE SYSTEM: INSIGHTS INTO REACTION COMPOSITION AND TRANSLATION EFFICIENCY

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ABSTRACT

The One-Pot PURE system for *in vitro* protein expression, which results from a co-culture and one-step purification of 36 essential proteins to support gene transcription and translation, can significantly improve the accessibility and affordability of PURE systems. However, replicating this protocol to match the productivity of the traditional PURE system can take considerable time and effort due to variability in the expression level of individual proteins. In this work, we observed unstable PURE protein expression in two *E. coli* protein expression strains, M15/pREP4 and BL21(DE3), and addressed this instability using catabolite repression. We identified key proteins whose concentration in the One-Pot PURE mixture significantly impacted the reaction's protein expression capacity. Compared to the original method using two *E. coli* protein expression strains, we found that consolidating all expression vectors onto one BL21 (DE3) strain led to more uniform cell growth at the time of protein induction, thereby improving the composition of critical translation initiation factors in the purified mixture for efficient translation. We also found that variations in commercial energy solution formulations could compensate for deficiencies in the One-Pot PURE protein composition. Additionally, our study revealed significant differences in the translation capacity of commercially available *E. coli* tRNAs, suggesting the potential of optimizing tRNA composition to improve protein translation. Taken together, this work highlights the intricate biochemical interplay influencing protein expression capacity in the One-Pot PURE system and presents strategies to improve its robustness and productivity.

KEYWORDS

PURE system, *in vitro* protein expression, cell-free transcription-translation, synthetic biology

INTRODUCTION

In vitro protein expression harnesses living cells' core gene expression machinery to allow transcription and translation to occur in a test tube reaction. The open nature of these expression platforms allows direct manipulation of the reaction environment. Applications of these platforms span high-throughput screening,[1–3] novel protein modifications,[4,5] interfacing biomolecules with synthetic materials,[6–9] on-demand biosensing[10–12] and biomanufacturing,[13,14] and understanding the rules of life by building it from scratch.[15–18]

Nearly all *in vitro* protein expression platforms can be categorized into two classes – crude lysate-based and reconstituted systems. Crude lysate-based expression systems use extracts from lysed cells, which retain most of the cell's proteome and metabolic pathways[19,20] and can thus support gene expression from a wide range of promoters and enable higher protein expression due to effective energy regeneration.[21] They can also be exceptionally affordable at approximately 3 cents per microliter of reaction.[22] However, the active endogenous metabolism and intact proteome can divert critical energy sources and metabolites to side reactions, interfering with the central reactions implemented in these systems.[20]

On the other hand, the PURE (Protein synthesis Using Recombinant Elements) system isolates the necessary transcription, translation, and energy regeneration proteins to support gene expression by protein purification and reconstitution. This creates a more defined reaction environment, enabling the construction of predictive models for protein expression,[23,24] allowing for facile removal and replacement of reaction enzymes for customization,[5] and maintaining a minimal reaction proteome toward a self-regenerating synthetic cell.[16] However, traditional PURE systems are expensive to purchase ($0.64 – 1.00 per microliter of reaction) and laborious to prepare in-house, posing a significant bottleneck in the wide adoption of PURE systems.

To address these limitations, the One-Pot PURE system developed by Lavickova and Maerkl offers a simplified alternative.[25] Instead of individually purifying each of the 36 PURE proteins, a co-culture of the 36 *E. coli* strains, each expressing a single protein, is grown, induced for protein expression, and purified in a single preparation. This approach significantly streamlines the PURE system preparation and delivers comparable protein yield to commercial PURE systems at a fraction of the cost ($0.10 per microliter of reaction).

Despite the promise of this method, replicating the One-Pot PURE protocol to achieve comparable productivity to conventional PURE systems still takes considerable time and effort. In this work, we document the sources of variability that result in less productive One-Pot PURE preparations, such as expression vector instability and sub-optimal PURE protein composition for effective protein translation. In addition to optimizing the PURE protein composition, we find that differences in the reaction's biochemical composition can significantly impact the translation rate and the final protein yield. Taken together, this work improves our current understanding of the factors influencing protein expression capacity in the One-Pot PURE system to enable its robust implementation across different labs and applications.

RESULTS AND DISCUSSION

Enhancing protein expression stability for the One-Pot PURE system through glucose-mediated catabolite repression

We began by replicating the One-Pot PURE system using plasmids deposited on Addgene and transforming them into the respective *E. coli* M15/pREP4 and BL21(DE3) expression strains. Proteins in pQE30 and pQE60 expression vectors were transformed into M15/pREP4 competent cells, and proteins in pET21 and pET15 expression vectors were transformed into BL21 (DE3) competent cells (**Figure 1A**).

Our initial monoculture protein expression assessment revealed that several protein expression vectors were unstable. Despite confirming plasmid sequences through whole plasmid sequencing, we observed a loss of expression in ten proteins after growing and inducing protein expression

from saved glycerol stocks (**Figure 1B, C**). No growth defect was observed for these non-expressing cells (**Figure SI.III.1**). This phenomenon was also observed in several expression strains directly obtained from a collaborator, with the loss of protein expression appearing in other strains, indicating the expression vector instability is a stochastic yet frequent event for PURE proteins (**Figure SI.III.2**).

The expression vector instability is problematic because a previously validated set of PURE protein expression strains can spontaneously lose protein expression ability after being saved to, and grown from, glycerol stocks. Without addressing this, subsequent preparations of the One-Pot PURE system can exhibit protein dropouts, leading to unproductive batches. Alternatively, each One-Pot PURE preparation will have to be made from fresh transformations of the 36 protein expression vectors, which is labor-intensive. Being able to stabilize the growth and expression of PURE proteins from glycerol stocks can significantly simplify the preparation workflow and facilitate the dissemination of these strains to other labs.

We hypothesize that leaky background protein expression from $P_{T7\text{-}lacO}$ and $P_{T5\text{-}lacO\text{-}lacO}$ promoters from BL21(DE3) and M15/pREP4 strains, respectively, can create growth burdens, leading to the loss of protein expression. This has been observed in another work, where background protein expression burden led to promoter mutations, resulting in the loss of protein expression.[5] To reduce background protein expression, we investigated whether catabolite repression, where the addition of a preferred carbon source (glucose), could upregulate the intracellular concentration of repressors (LacI) that block secondary carbon source utilization. [26]

With upregulated LacI repressor production and reduced background protein expression from $P_{T7\text{-}lacO}$ and $P_{T5\text{-}lacO\text{-}lacO}$ promoters, we expect more stable plasmid maintenance and protein expression. Indeed, our results showed that by supplementing 1% w/v glucose to the growth media until the final culture, the PURE protein expression was stably maintained even after five passages from the seed glycerol stock (**Figure 1D**). For strains grown in catabolite repression media, we also observed improved cell growth and higher protein expression from fresh transformations of the 10 protein expression plasmids previously found to lose protein expression (**Figure SI.III.3**). All subsequent One-Pot PURE batches are grown with LB media supplemented with 1% w/v glucose.

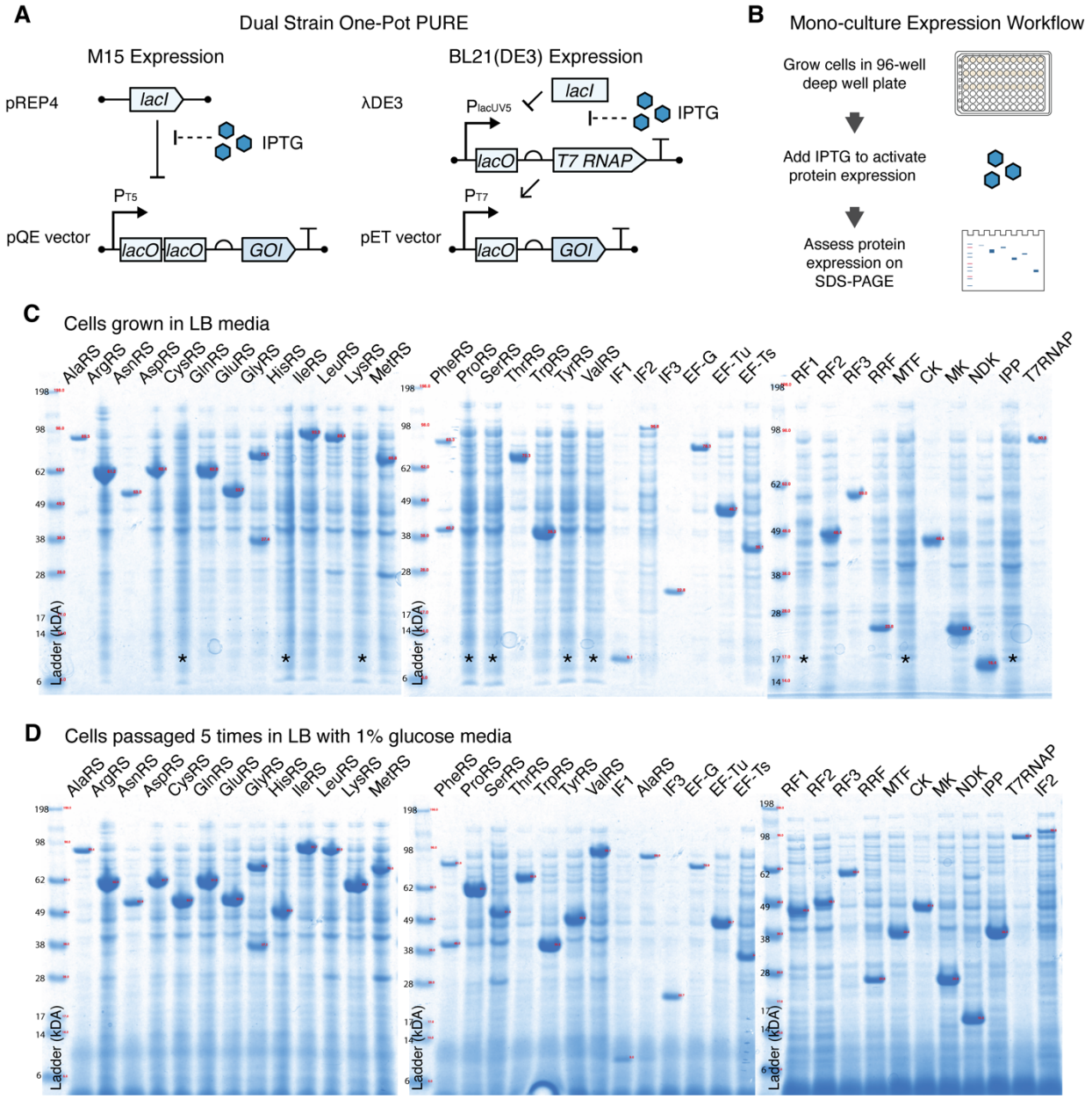


Figure 1 (A) Schematic of protein expression and regulation in *E. coli* strains M15/pREP4 and BL21(DE3) for Dual Strain One-Pot PURE systems. Each gene of interest is expressed under the control of either the P_{T5} or P_{T7} promoter followed by lacO operator site(s). In the M15/pREP4 expression strain, the pREP4 plasmid constitutively expresses the *lac* inhibitor, LacI, which represses protein expression by binding to the lacO sites. In BL21(DE3), endogenous LacI represses the production of T7 RNA polymerase (RNAP) and gene expression by binding to the lacO sites. Addition of the IPTG inducer de-represses LacI inhibition and activates gene expression. **(B)** Schematic of monoculture protein expression workflow. Cells were grown in 96-well deep-well plates, and protein expression was activated by adding 0.1 mM IPTG inducer. Following cell growth, protein expression was assessed via SDS-PAGE. **(C)** Monoculture protein expression assessment from cells grown in LB media. Ten strains (designated by *) were found to carry the protein expression plasmid but lacked the protein expression capability. **(D)** Stable protein expression from cells grown in LB with 1% w/v glucose after 5 passages, with all PURE proteins visibly expressed.

Offsets in the Dual Strain One-Pot PURE protein composition contribute to low protein production.

After resolving the protein expression issue, we proceeded to replicate the original One-Pot PURE system with BL21(DE3) and M15/pREP4 expression strains (Dual Strain One-Pot PURE) following the established protocol.[27] To assess the system's transcription and translation capacity, we used a reporter plasmid with a malachite green aptamer (MGA) to approximate mRNA concentration and destabilized enhanced green fluorescent protein (deGFP) to report on the protein yield (**Figure 2A**). Surprisingly, while the system produced a high amount of mRNA transcripts of up to 6 μM, the protein expression was capped at approximately 1.5 μM for deGFP (**Figure 2B**) for two batches of the One-Pot PURE system. This protein yield is less than a third of the reported One-Pot PURE reaction yield.[25,27] We also note the high initial malachite green mRNA concentration may come from rapid mRNA transcription that began before the microwell plate was placed into the plate reader.

Given that we have confirmed all strains are expressing the One-Pot PURE proteins with mono-culture expression assays, we hypothesized that there might be poor cell growth or protein expression in the co-culture environment that was not captured by the monoculture assays. Resolving the purified One-Pot PURE protein mixture on a gel, as done in the monoculture assays, would be challenging due to the similarity in molecular weight of many proteins. Therefore, we opted for a targeted proteomics approach to characterize the relative abundance of these proteins.

Using liquid chromatography with tandem mass spectrometry (LC-MS/MS), we mapped the detected peptides to protein abundances. After scaling for their concentrations in reactions, we compared individual proteins' relative abundances in the Dual Strain One-Pot PURE mixture to protein abundances in commercial PURE systems (**Figure 2C**). Our analysis revealed a consistent deficiency in 7 proteins in Dual Strain One-Pot PURE systems compared to the two commercial PURE systems ($PURE_A$ and $PURE_B$). These proteins are alanyl-tRNA synthetase (AlaRS), initiation factor 2 (IF2), initiation factor 3 (IF3), elongation factor-G (EF-G), elongation factor-Tu (EF-Tu), elongation factor-Ts (EF-Ts), and creatine kinase (CK).

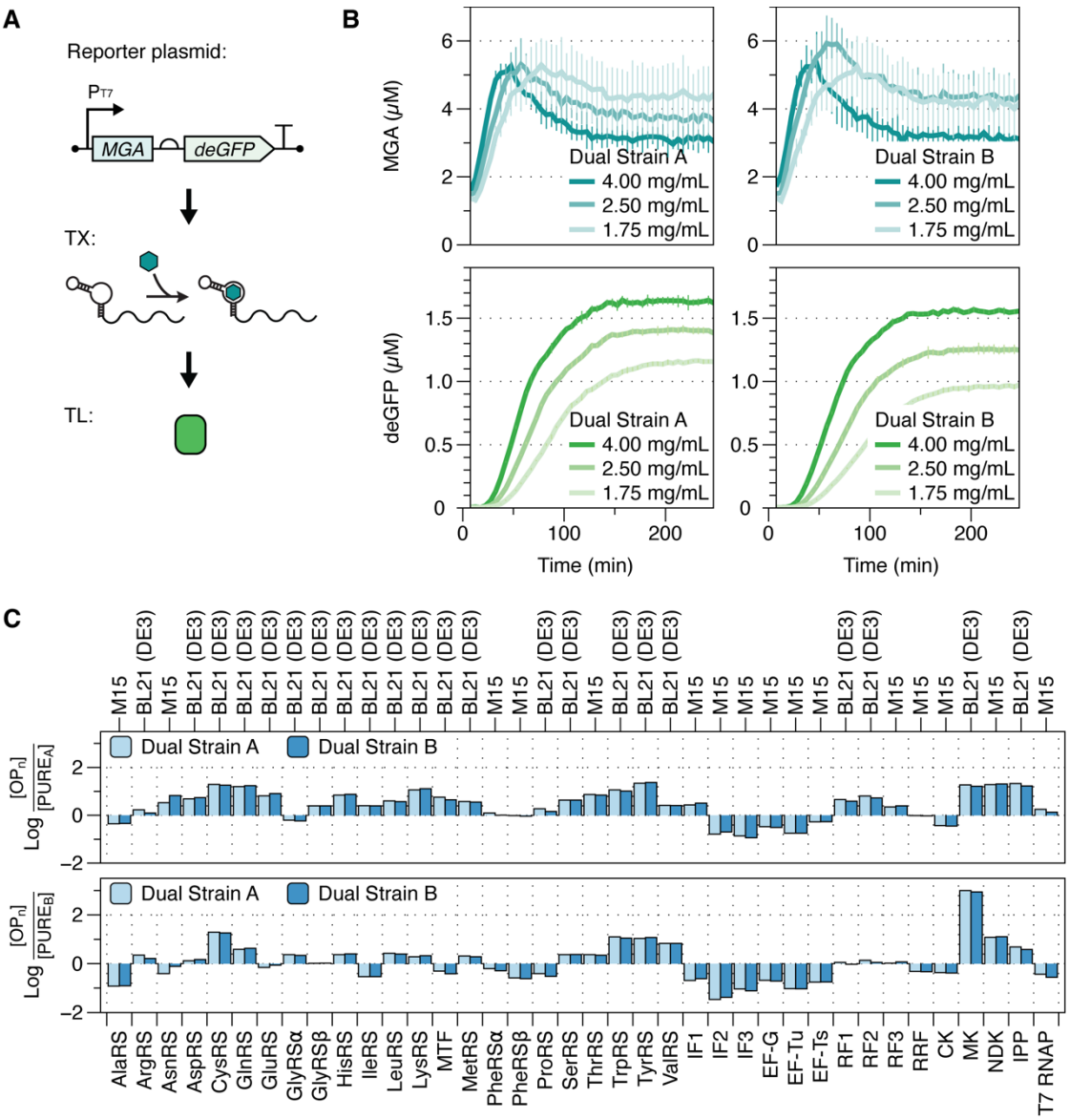


Figure 2: (A) Schematic of the reporter plasmid used to assess transcription and translation activity, using mRNA transcript with a malachite green aptamer (MGA) and coding sequences for destabilized enhanced green fluorescent protein (deGFP) expression. The fluorescence was monitored for malachite green dye-bound MGA and folded deGFP. **(B)** The productivity of two batches of Dual Strain One-Pot PURE systems (Dual Strain A and Dual Strain B) in mRNA transcription and protein translation, measured via MGA and deGFP, respectively. The One-Pot PURE protein concentration in the reactions was varied from 1.75 to 4 mg/mL to optimize yield. The plots represent the average of reaction triplicates, with error bars indicating the standard deviations of reaction triplicates. **(C)** Comparison of Dual Strain One-Pot PURE protein abundances to protein abundances in two commercial PURE systems after scaling by their concentrations in the reaction.

Enhancing One-Pot PURE protein composition and expression capacity with a Single-Strain One-Pot PURE system

Since all seven of the consistently deficient proteins are expressed in the *E. coli* M15/pREP4 expression strain, we investigated whether these cells had growth deficiencies. From the monoculture cell growth data, we observed that M15/pREP4 cells exhibited slower growth rates during and after IPTG induction for protein expression (**Figures SI.III 1 and 2**). As a result, we transferred all M15/pREP4-expressed proteins to BL21(DE3) cells to achieve more uniform growth during and after IPTG induction. We note that a caveat in this single-strain version is that the T7 RNAP needs to be removed from the One-Pot PURE co-culture due to the anticipated genetic instability of having a P_{T7} promoter expressing T7 RNAP. As a result, we opted to purify and add T7 RNAP into One-Pot PURE reactions separately (see **MATERIALS AND METHODS**). Alternatively, T7 RNAP could be purchased commercially.

Our results showed that five out of the 16 proteins previously expressed in the M15/pREP4 cells exhibited at least a 30% improvement in expression when grown and expressed in BL21(DE3) cells, as determined by monoculture growth and protein gel assessment (**Figure SI.III 4**). This enhancement is also evident in the One-Pot PURE co-culture. Compared to the Dual Strain system, the purified PURE protein mixture from the Single Strain system has a slightly higher concentration of translation factors (24% and 26% for Dual Strain and Single Strain, respectively, **Figure 3B**). In resolving the individual protein composition using mass spectrometry, we found that translation initiation factors 2 and 3 (IF2 and IF3) and creatine kinase (CK) involved in energy regeneration (**Figure 3A**) are present at significantly higher concentrations in Single Strain One-Pot PURE. An Individual protein composition comparison of Single Strain One-Pot PURE to commercial PURE systems is also provided in **Figure SI.III 5**.

We next compared the productivity of the Dual Strain and Single Strain versions of the One-Pot PURE system. Using only the energy solution from commercial vendors and replacing PURE proteins with One-Pot PURE proteins, we observed consistent high protein production with the Single Strain One-Pot PURE system (**Figure 3C-D**).

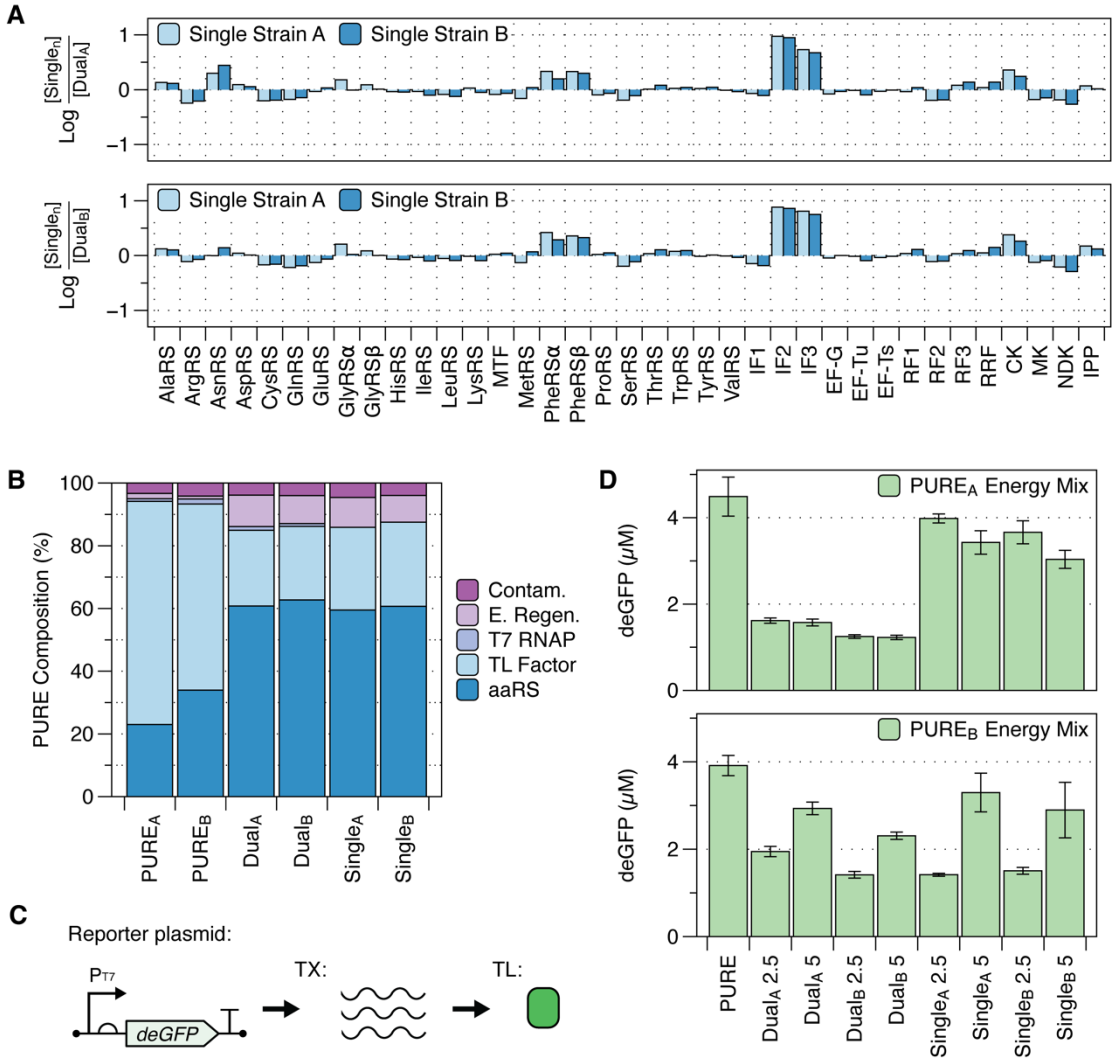


Figure 3: (A) Comparison of the relative abundance of individual One-Pot PURE proteins in the Single Strain and Dual Strain systems. Initiation factors 2 and 3 (IF2, IF3) show the most notable increase in composition in the Single Strain One-Pot PURE system. Previously under-expressed aminoacyl tRNA-synthetases (AlaRS and PheRS) and creatine kinase (CK) also increased in composition in the Single Strain One-Pot PURE system. **(B)** Composition of commercial PURE proteins, Dual Strain One-Pot PURE proteins, and Single Strain One-Pot PURE proteins grouped by protein classes: aminoacyl tRNA synthetase (aaRS), translation factors (TL factors), T7 RNAP, energy regeneration (E Regen.). Detected proteins not part of the PURE proteins are categorized as contaminants (Contam.). Ribosomal proteins are excluded from analysis. **(C)** Schematic of the reporter plasmid P_{T7}-UTR1-deGFP expressed in PURE reactions to assess system productivity. **(D)** Productivity of Dual Strain and Single Strain One-Pot PURE reactions assembled with two commercial energy mixes. $PURE_A$ and $PURE_B$ are control reactions with 5 nM PT7-UTR1-deGFP plasmids assembled according to the manufacturer's instructions. $Dual_n$ 2.5 and 5 designate varying batches of Dual Strain One-Pot PURE proteins added at 2.5 and 5 mg/mL. Likewise, $Single_n$ 2.5 and 5 designate varying batches of Single Strain One-Pot PURE proteins added at 2.5 and 5 mg/mL. The bar graphs represent the average terminal protein yield of reaction triplicates, with error bars indicating the standard deviations of reaction triplicates.

205 ***High-yield protein expression from One-Pot PURE requires a balance between the rate of***
206 ***protein translation and energy regeneration.***

207 The differences in productivity between the Dual Strain One-Pot PURE system in two commercial
208 PURE energy mixes, referred to as $PURE_A$ and $PURE_B$, are particularly interesting. It appears
209 that the $PURE_B$ energy mix can support more One-Pot PURE proteins in a reaction as a strategy
210 to compensate for the lower concentration of translation factors in the purified mixture (**Figure**
211 **3D**). In reactions with the $PURE_A$ and homemade energy mixes, adding more One-Pot PURE
212 proteins to the reaction decreases the final protein yield after an optimal concentration.

213 Comparing the protein translation rates of both types of One-Pot PURE systems to commercial
214 PURE systems provides valuable insights (**Figure 4** and **Figure SI.III 6-7**). In the $PURE_A$ energy
215 solution, the translation rates of the Dual Strain and Single Strain One-Pot PURE systems
216 behaved as expected (**Figure 4**). Deficiencies in translation proteins in the Dual Strain One-Pot
217 PURE system contributed to a lower translation rate over the course of the reaction. With more
218 protein translation machinery, the Single Strain One-Pot PURE system could match or even
219 exceed the translation rate of commercial $PURE_A$ proteins. However, higher translation rate peaks
220 led to steeper drops in translation rate shortly after, as observed for the Single Strain One-Pot
221 PURE system with the $PURE_A$ energy solution.

222 In the $PURE_B$ energy solution, when both the Dual Strain and Single Strain One-Pot PURE
223 proteins are added to 5 mg/mL, both systems exhibited comparable protein expression levels
224 despite the deficiency in translation factors found in the Dual Strain One-Pot PURE proteins
225 (**Figure 4**). It is important to note that the contribution to these differences in translation rate
226 comes from the energy solution alone (amino acids, NTPs, tRNA, creatine phosphate, and other
227 co-factors). This result suggests that an optimal preparation of the energy solution could
228 compensate for variations in the One-Pot PURE protein composition.

229 We also observed that when the translation rate from the One-Pot PURE system exceeded that
230 of commercial PURE proteins, a steep drop in translation rate soon followed (**Figure 4**). This
231 suggests that while faster translation can occur, it can also significantly deplete the available
232 energy molecules (ATP and GTP) critical for translation initiation and elongation reactions. A fast
233 translation rate that surpasses the system's energy regeneration capacity can negatively impact
234 the final protein yield by stalling protein translation, potentially resulting in premature translation
235 termination and lower apparent protein production.

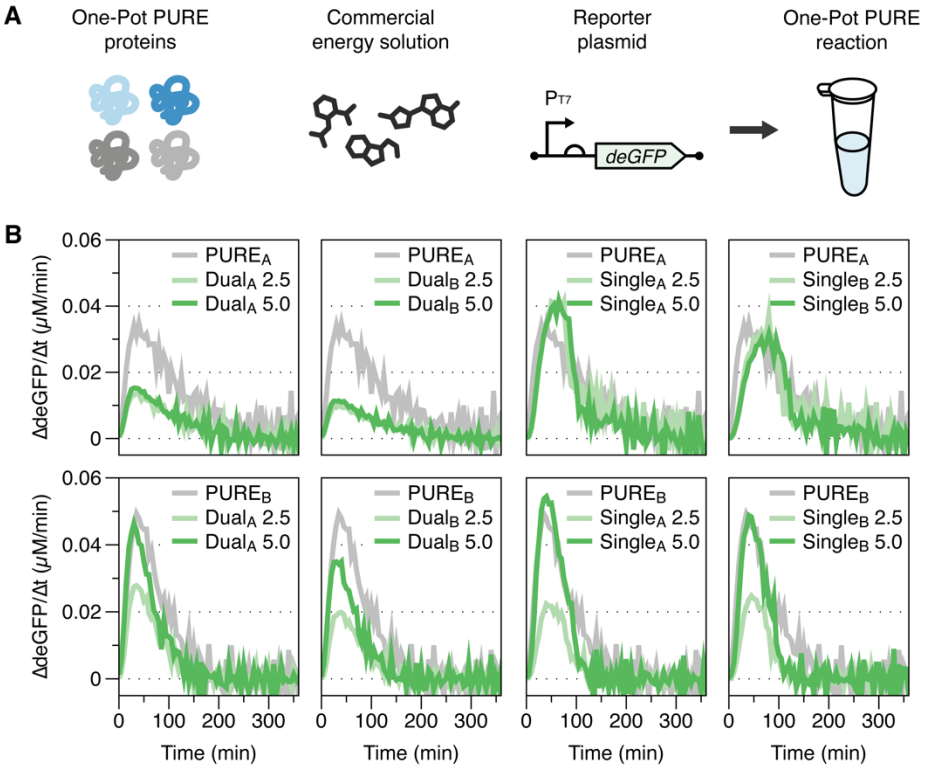
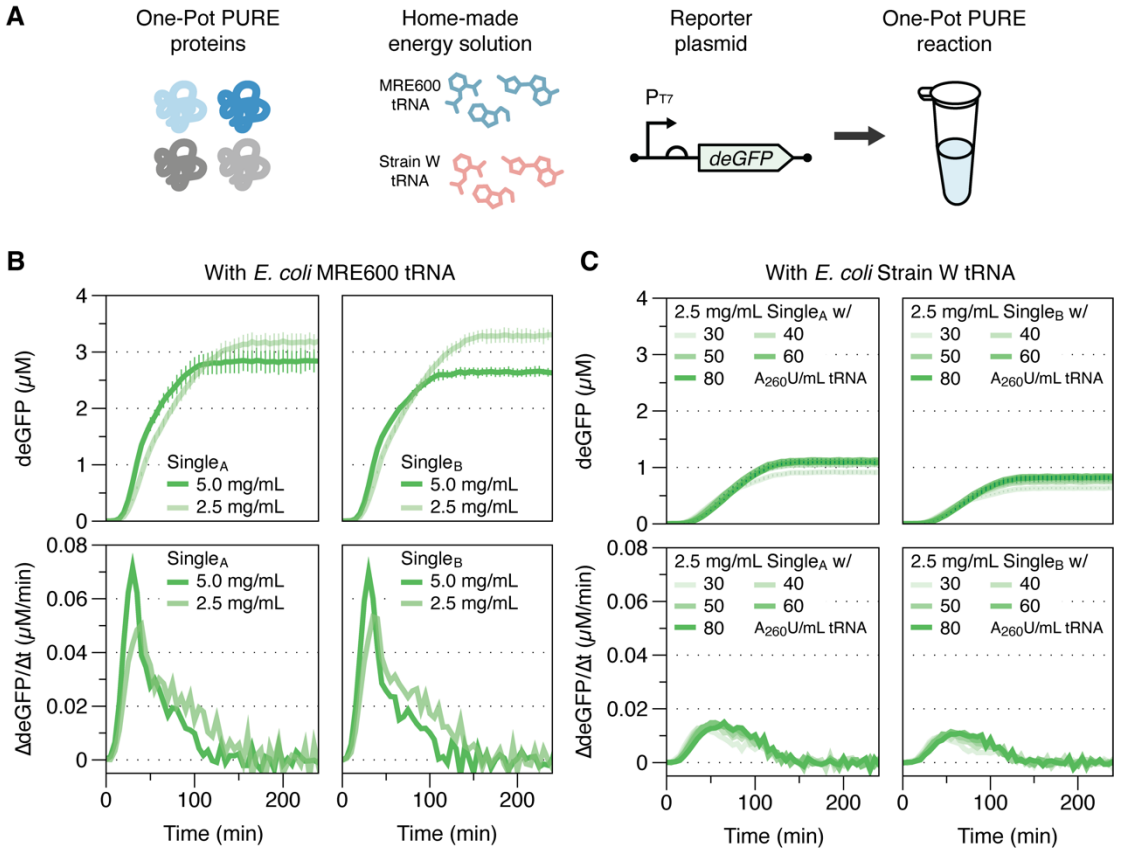


Figure 4: (A) Schematic of One-Pot PURE reaction composition using in-house preparations of Dual Strain and Single Strain One-Pot PURE proteins with commercial energy solution according to manufacturers' protocols and 5 nM of P_{T7}-UTR1-deGFP. In the case of Single Strain One-Pot PURE reactions, 0.5 μM of purified T7 RNAP was added. **(B)** Comparison of protein translation rate for various preparations of One-Pot PURE proteins (added to the reaction at 2.5 or 5.0 mg/mL) in two commercial energy mixes. Plots represent the average of reaction triplicates. Time traces of deGFP production are provided in the **Supplementary Figures SI.III. 6-7**.

243 ***E. coli tRNA composition critically influences One-Pot PURE reaction productivity.***

244 With an optimized Single Strain One-Pot PURE system, we proceeded to validate the protein
245 expression capacity with an in-house prepared energy solution. Two commercial sources of *E.*
246 *coli* tRNA were tested, one from *E. coli* strain MRE600 and one from *E. coli* strain W. The energy
247 solution prepared using *E. coli* MRE600 tRNA exhibited a high protein translation rate and final
248 protein yield (**Figure 5B**). When we replaced tRNA in the energy solution with *E. coli* Strain W
249 tRNA, a significant decrease in protein translation rate and yield was observed(**Figure 5C**).
250 Increasing *E. coli* Strain W tRNA concentration did not increase protein expression.

251 This finding is significant because the complexity and composition of tRNAs can be overlooked
252 when preparing the energy solution for PURE reactions. Differences in codon usage among
253 various *E. coli* strains can lead to unexpected deficiency in tRNA associated with some codons.
254 Without prior knowledge of the deficient tRNA, it can be difficult to codon-optimize the protein for
255 effective expression, leading to low protein yield despite having an otherwise productive system.



256

257 **Figure 5: (A)** Schematic of One-Pot PURE reaction composition using in-house prepared energy solutions with
258 commercial tRNAs from *E. coli* MRE600 and *E. coli* Strain W. Each reaction contains 5 nM of P_{T7}-UTR1-deGFP to
259 assess protein production. **(B)** Comparison of deGFP protein production and its rate of production for two batches of
260 Single Strain One-Pot PURE proteins (added to the reaction either at 2.5 or 5.0 mg/mL) in energy solution with 22
261 A_{260}U/mL *E. coli* MRE600 tRNA. Error bars represent standard deviations of reaction triplicates. Protein translation rate
262 plots represent the average of reaction triplicates. **(C)** Comparison of deGFP protein production and its rate of
263 production for two batches of Single Strain One-Pot PURE proteins (added to the reaction at 2.5 mg/mL) in energy
264 solution with increasing *E. coli* Strain W tRNA activity units (30 – 80 A_{260}U/mL). The plots represent the average of
265 reaction triplicates, and the error bars represent standard deviations of reaction triplicates. Protein translation rate plots
266 represent the average of reaction triplicates.

CONCLUSION

In this work, we identified several important factors that affect the expression capacity of the One-Pot PURE system. We improved the stability of One-Pot PURE protein expression through glucose catabolite repression. We consolidated all protein expression vectors from the original dual strain design to one *E. coli* BL21(DE3) strain, increasing the expression levels of critical translation initiation and energy regeneration proteins and, consequently, the productivity of the One-Pot PURE reaction. Our characterization of both dual and single-strain One-Pot PURE proteins in two commercial PURE energy solutions revealed that optimizing biochemical compositions could overcome deficiencies in translation capacity. Lastly, our in-house energy mixture preparation revealed the critical role of tRNA composition in determining the translation capacity of PURE reactions.

These findings showcase that the gene expression capacity of the PURE system is influenced by both protein and biochemical factors. At the protein level, we found that translation initiation factors need to be at a higher concentration in the One-Pot PURE protein mixture to support effective protein production (**Figure 3**). This suggests the possibility of compensating for a sub-optimal One-Pot PURE protein preparation with a purified mixture of these critical translational proteins.

At the biochemical level, we found that energy mix composition significantly impacts the One-Pot PURE translation rate and the final protein yield. Using the $PURE_A$ energy mix, the reaction proceeded with a slower protein translation rate and took over 6 hours to stabilize to the final deGFP concentration (**Figure SI.III 6**). This suggests that there may be a translation rate-limiting step imposed by the $PURE_A$ energy mix, and this limitation can make One-Pot PURE proteins with sub-optimal protein composition proceed even more slowly, resulting in lower protein yield. On the other hand, the $PURE_B$ energy mix supports a faster translation rate overall, with reactions completed in under 3 hours (**Figure SI.III 7**). This suggests the rate-limiting step observed in the $PURE_A$ energy mix may be removed in $PURE_B$ to deliver a high protein yield with a shorter reaction lifetime. Interestingly, the reaction bottleneck comes from the PURE protein concentration. When more PURE proteins were added to the $PURE_B$ energy mix, a faster translation rate and a higher protein yield were observed.

Additionally, we show that commercial preparations of *E. coli* tRNA sourced from strain MRE600 and strain W significantly impact effective protein translation (**Figure 5**). Although MRE600 is a highly preferred source for *E. coli* tRNA due to the strain's low RNase I activity,[28] there is no apparent reason behind tRNA obtained from strain W (a safe and fast-growing strain utilizing sucrose as an industrially preferred carbon source)[29] exhibiting sub-optimal translation capacity. This highlights a critical limitation and source of variability in most cell-free systems (lysate-based and PURE), which is that *E. coli* tRNA is also a complex mixture and exhibits differential concentration profiles across growth stages and host strains.[30,31] For robust control over both One-Pot PURE reaction and potentially lysate-based cell-free expression systems, a promising approach can be developing an optimal tRNA composition.[32]

Taken together, this work seeks to obtain a comprehensive understanding of the factors influencing the gene expression capacity of the One-Pot PURE system. By addressing both protein and biochemical composition, our findings offer valuable insights into optimizing not only One-Pot PURE systems but also extending to lysate-based cell-free protein synthesis, paving the way for more efficient and reliable applications in synthetic biology and biotechnology.

MATERIALS AND METHODS

Escherichia coli strains and plasmids

E. coli BL21(DE3) (Thermo Scientific) and M15/pREP4 (Qiagen) strains were used for protein expression. **Supplementary Information I** contains the Addgene catalog numbers and plasmid sequences for Dual-Strain One-Pot. To establish the Single Strain One-Pot, all proteins were amplified via Gibson Assembly and placed onto the pET21a backbone. All sequences of cloned proteins transplanted onto pET21a are provided in **Supplementary Information I**.

Sequence-verified plasmids (Primordium Labs, now Plasmidsaurus) were transformed into their respective BL21(DE3) and M15/pREP4 expression strains and plated on Luria Broth (LB) Agar plates with 100 μg/mL carbenicillin and 1% glucose. An inoculation loop was used to scrape multiple colonies to inoculate overnight culture in LB with 100 μg/mL carbenicillin and 1% glucose to obtain the population expression average. To facilitate inoculation of subsequent final cultures, 250 μL of overnight cultures were saved into frozen glycerol stocks with 250 μL of 50% glycerol in 1.3 mL 96 well plates (NEST Biotech). The glycerol stock plate was sealed with an aluminum film and stored at -80 ˚C.

Sequences of plasmid DNA used for reporter protein expression are provided in **Supplementary Information I**. All plasmid DNA was prepared using the NucleoBond Xtra Midi kit (Macherey-Nagel) and eluted in nuclease-free water.

Monoculture Expression assay for One-Pot PURE proteins

The protocol was adapted from Grasemann *et al.*[27] with a few modifications. An overnight culture of One-Pot PURE protein expression strains was inoculated either from freshly transformed cells grown on LB agar plates with carbenicillin (without or with 1% w/v glucose to exert catabolite repression) or from frozen glycerol stocks using a cryo-replicator (Enzyscreen, CR1000). The overnight culture was grown in 96-well deep-well plates sealed with a Breathe-Easier membrane (Diversified Biotech) at 37 ˚C and 1000 rpm.

The next morning, a fresh starter culture was prepared using 4 μL of the overnight cells and 396 μL of LB with carbenicillin. OD600 was monitored at the time of 0.1 mM IPTG induction to initiate protein expression after 2-2.5 hrs of growth and 3 hours after protein expression. 10 μL of cells expressing One-Pot PURE proteins were mixed with Lammeli Buffer (Bio-Rad) and boiled at 95 ˚C for 10 minutes, and 10 μL of the mixture was loaded onto Bolt™ 4-12% Bis-Tris Protein Gels (Invitrogen) along with protein ladders (SeeBlue™ Plus2 Pre-stained Protein Standard, Invitrogen). Samples were run at 150 kV, 400 mAmp for 30 minutes with NuPAGE MES SDS buffer (Invitrogen).

The gel was stained with SimplyBlue Safe Stain (Invitrogen) for 1 hour and de-stained in water overnight before imaging on a ChemiDoc Imager (Bio-Rad) or a GelDoc Go Imager (Bio-Rad). The obtained protein gel images were analyzed using the accompanying software Image Lab 6.1 for Mac (Bio-Rad).

349 ***One-Pot PURE reaction assembly and data acquisition***

350 Detailed methods describing the preparation of One-Pot PURE protein mixture and energy
351 solution are provided in **Supplementary Information II**.

352 All One-Pot reactions were assembled on ice using low protein-binding microcentrifuge tubes
353 (Eppendorf). Each reaction is 5 μL in total volume and consists of 2 μM *E. coli* Ribosome (NEB),
354 5 nM reporter protein expression plasmids (either P_{T7}-MGA-UTR1-deGFP or P_{T7}-UTR1-deGFP),
355 2.5x Energy Mix (125 mM HEPES-KOH pH 7.6, 250 mM Potassium L-Glutamate, 5 mM ATP and
356 GTP at pH 7.5, 2.5 mM UTP and CTP at pH 7.5, either 56 A_{260}U/mL for *E. coli* MRE600 tRNA or
357 75 – 200 A_{260}U/mL for *E. coli* W tRNA, 50 mM Creatine Phosphate, 2.5 mM TCEP, 50 μM Folinic
358 Acid, 5 mM Spermidine, and 0.75 mM amino acids mixture at pH 7.5), 1.75 – 5 mg/mL of One-
359 Pot PURE proteins and 0.5 – 1 μM T7 RNAP (only for Single Strain One-Pot). For reactions using
360 malachite green aptamer (MGA) fluorescence as a proxy for mRNA transcripts, 10 μM of
361 malachite green dye was added to the reaction.

362 Following assembly, 5 μL of each reaction was pipetted onto a low-volume 384-well microplate
363 with a non-binding surface (Corning), and the reaction trajectory was measured every 5 minutes
364 using the BioTek Synergy Plate Reader at 37 ˚C. Fluorescence for deGFP was measured at
365 485/515 nm excitation/emission wavelengths at Gain 61, and fluorescence for malachite green
366 aptamer was measured at 610/650 nm excitation/emission wavelengths at Gain 150. The
367 fluorescence data for MGA and deGFP were calibrated to protein concentration data using
368 synthesized MGA RNA and deGFP protein standards, provided in **Supplementary Information**
369 **III**, **Figure SI.III.8**.

370 ***Acquisition and analysis of proteomics data***

371 A brief summary of LC-MS/MS workflow is provided below. Detailed methods are provided in
372 **Supplementary Information II**.

373 LC-MS/MS analyses of the digested peptides were performed on an EASY-nLC 1200 (Thermo
374 Fisher Scientific) coupled to a Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer
375 (Thermo Fisher Scientific). MS2 fragmentation spectra were searched with Proteome Discoverer
376 SEQUEST (version 2.5; Thermo Scientific, Waltham, MA) against an *in silico* tryptic digested *E.*
377 *coli* BL21 (DE3) and *E. coli* M15 database. The relative abundance of parental peptides was
378 calculated by integrating the area under the curve of the MS1 peaks.

379 The proteomics data presented in this manuscript are from the same batch of LC-MS/MS run for
380 results to be comparable. The relative protein abundances for each sample are first normalized
381 by scaling the total sample protein abundance with the average total protein abundance for all
382 samples. The proteins involved in the PURE system were extracted from the entire proteome
383 dataset and scaled by their composition in PURE reactions (manufacturer's recommended
384 compositions for $PURE_A$ and $PURE_B$, 4 mg/mL for Dual Strain One-Pot PURE batches A and B
385 in **Figure 2**, and 2.5 mg/mL for all Dual- and Single Strain One-Pot PURE batches A and B in
386 **Figure 3**)

AUTHOR CONTRIBUTIONS

Conceptualization: Y.Z., R.M.M., P.S.F., T.C.; *Investigation:* Y.Z., Y.Q., M.D.; *Analysis:* Y.Z., Y.Q., Z.A.M.; *Writing – Original Draft:* Y.Z.; *Writing – Review and Editing:* Y.Z, M.D., R.M.M., P.S.F., Y.Q., T.C., Z.A.M.; *Visualization:* Y.Z., Z.A.M.; *Supervision:* R.M.M., P.S.F., T.C.

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CONFLICT OF INTEREST

R.M.M. has a financial stake in Tierra Biosciences, a private company that uses bacterial lysate-based cell-free technologies for protein expression and screening.

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