Optimizing Cell-Free Protein Expression in the One-Pot PURE System

Insights into reaction composition and translation efficiency

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Presented at the 13th ICBE, January 7th, 2025, Houston, TX.

[1] California Institute of Technology | [2] Imperial College London

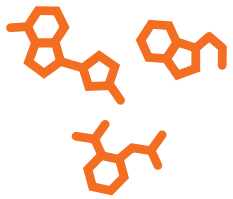
Caltech

Cell-free expression system is a powerful tool in breadboarding biomolecular designs

Cell-free expression system

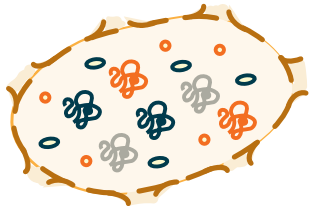
Genetic program

Gene expression machinery



Biochemical building blocks

Lysate-based cell-free system



The cell's entire soluble proteome

PURE-based cell-free system

Protein synthesis **U**sing **R**ecombinant **E**lements

1 Transcription factor

21 AA-tRNA synthetases

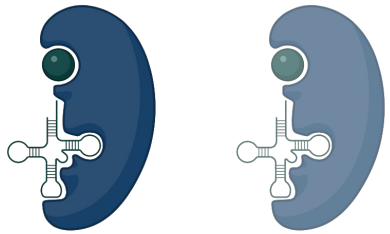
10 Translation factors

4 Energy cycling factors

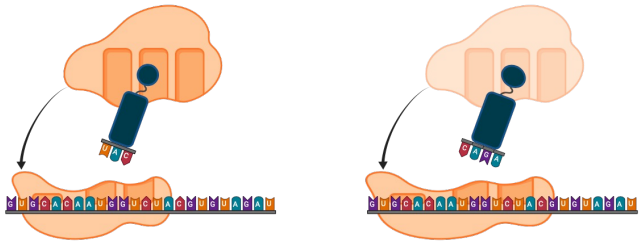
1 Ribosome complex

The PURE system can enable more biomolecular design possibilities over lysate-based cell-free systems

Replace aaRS for ncAA incorporation



Replace ribosome for quadruplet codon usage



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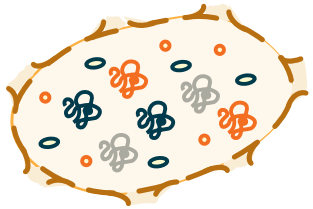

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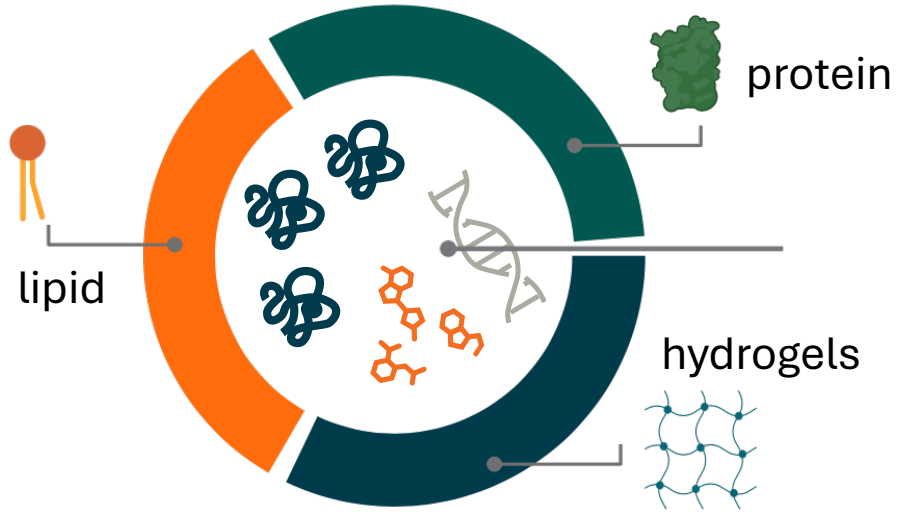
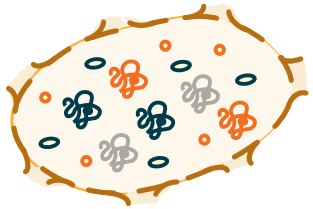
PURE system's minimal nature also makes it a desirable "cytosol" for synthetic cells

Synthetic cells – polymer container encapsulating biomolecules to execute cell-like functions



The minimal nature of PURE can be a desirable starting place to establish the "operating system" of synthetic cells

Lysate-based cell-free system



The cell's entire soluble proteome

PURE-based cell-free system

Protein synthesis **U**sing **R**ecombinant **E**lements

1 Transcription factor

21 AA-tRNA synthetases

10 Translation factors

4 Energy cycling factors

1 Ribosome complex

EXCEPT, making PURE system is highly labor-intensive
The One-Pot PURE system offers a streamlined solution

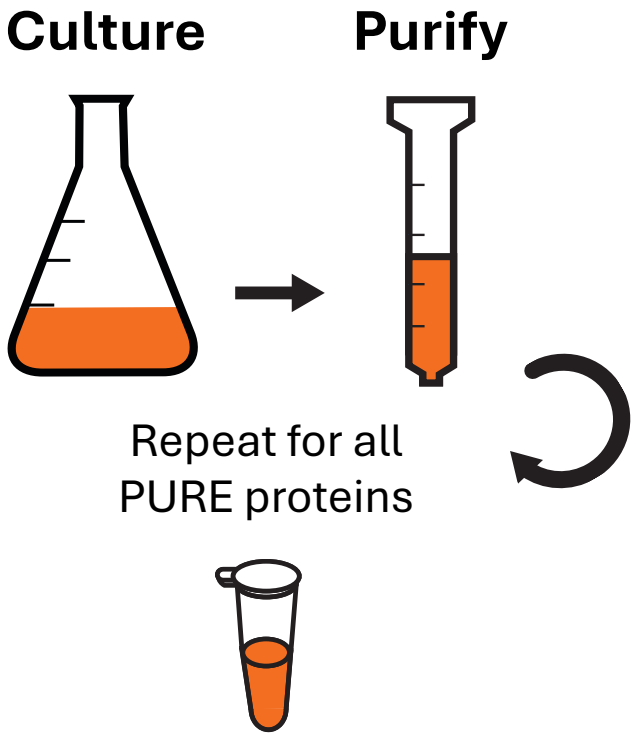
Machinery in the PURE system

- 1 Transcription factor
- 21 AA-tRNA synthetases
- 10 Translation factors
- 4 Energy cycling factors
- Ribosome complex *

*purified separately

Traditional PURE

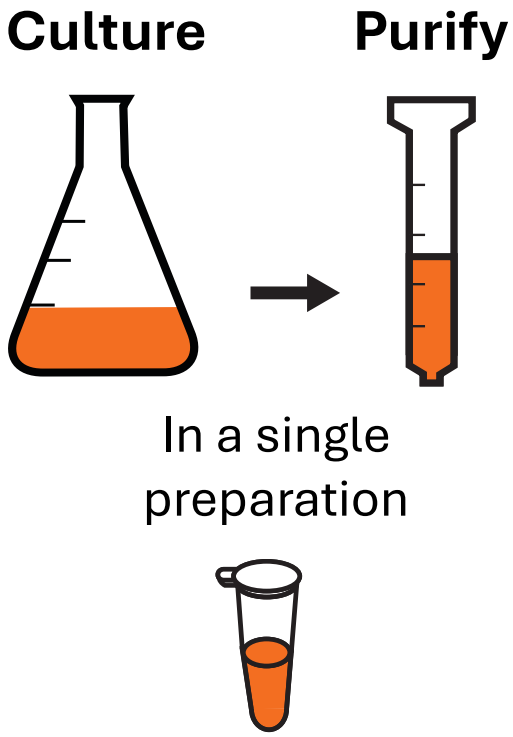
Shimizu *et al.*, 2001



~2 weeks to PURE

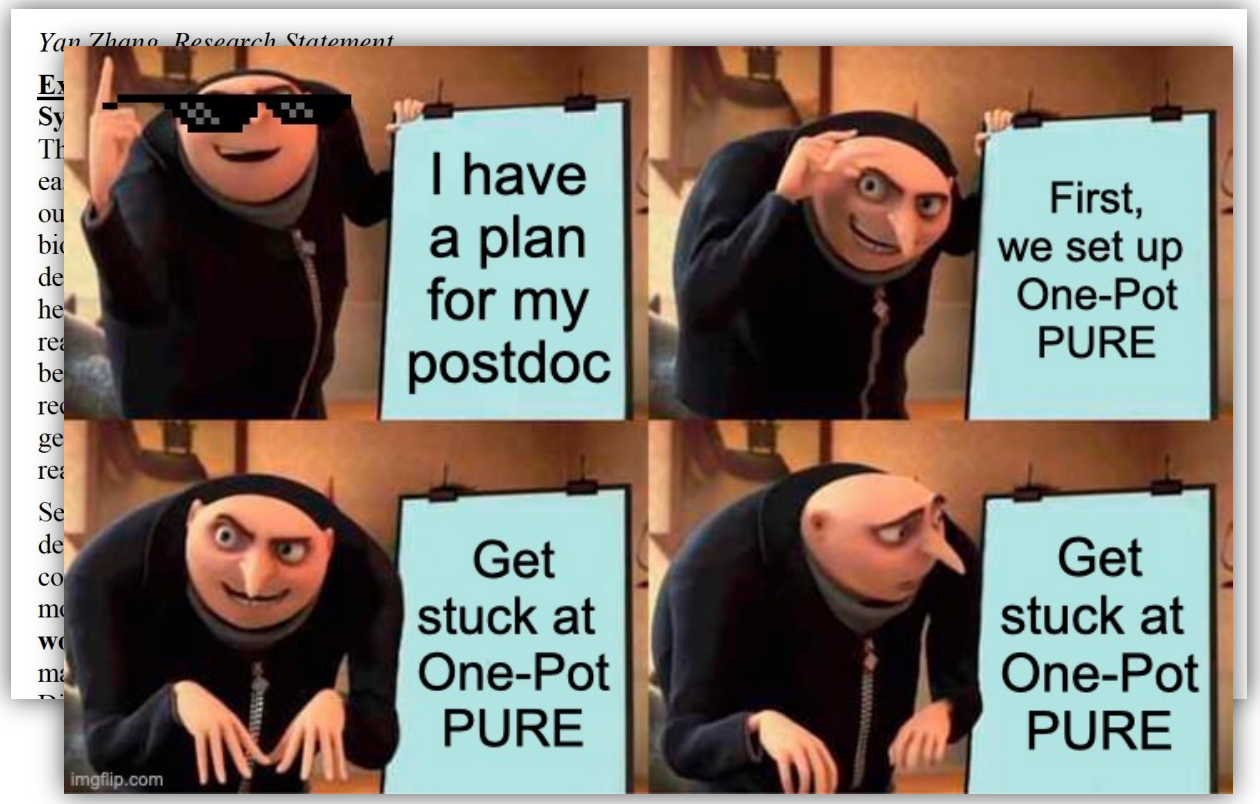
One-Pot PURE

Lavickova and Maerkl., 2019



~3 days to PURE

The One-Pot PURE appeared to be a better approach to set up synthetic cells for Yan's postdoc training plan



Imageflip.com, 2025

Step 1: Set up the One-Pot PURE system in the Murray Lab

Step 2: Encapsulate One-Pot PURE into synthetic cells

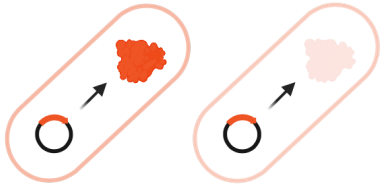
Step 3: Develop task-specialized synthetic cells

Step 4: Get results and publish

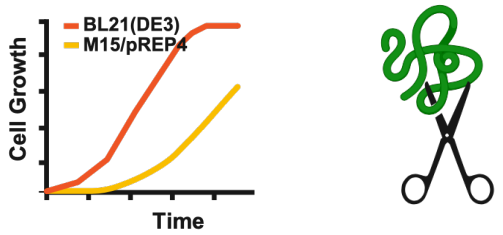
Overview of this talk:

Unforeseen short-comings with the One-Pot PURE system and mitigating strategies

Loss of PURE protein expression



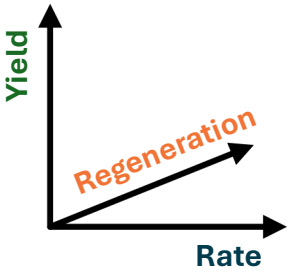
Slow growth and proteolysis in original expression strain



The reaction's biochemical composition strongly influences the system's gene expression capacity

Some energy mixes are robust to a suboptimal PURE

A balanced reaction rate is key to higher protein yield



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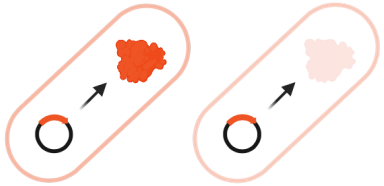

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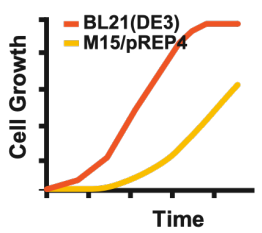

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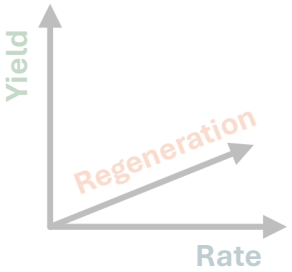
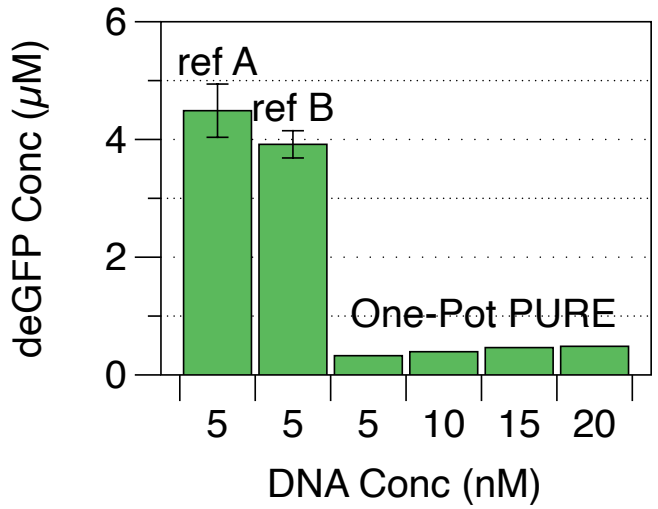


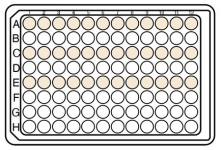
Unstable PURE protein expression led to spontaneous protein dropouts in the co-culture

First batch of One-Pot PURE



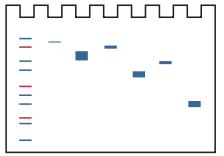
The productivity of **One-Pot PURE is 10 times lower** than that of commercial systems.

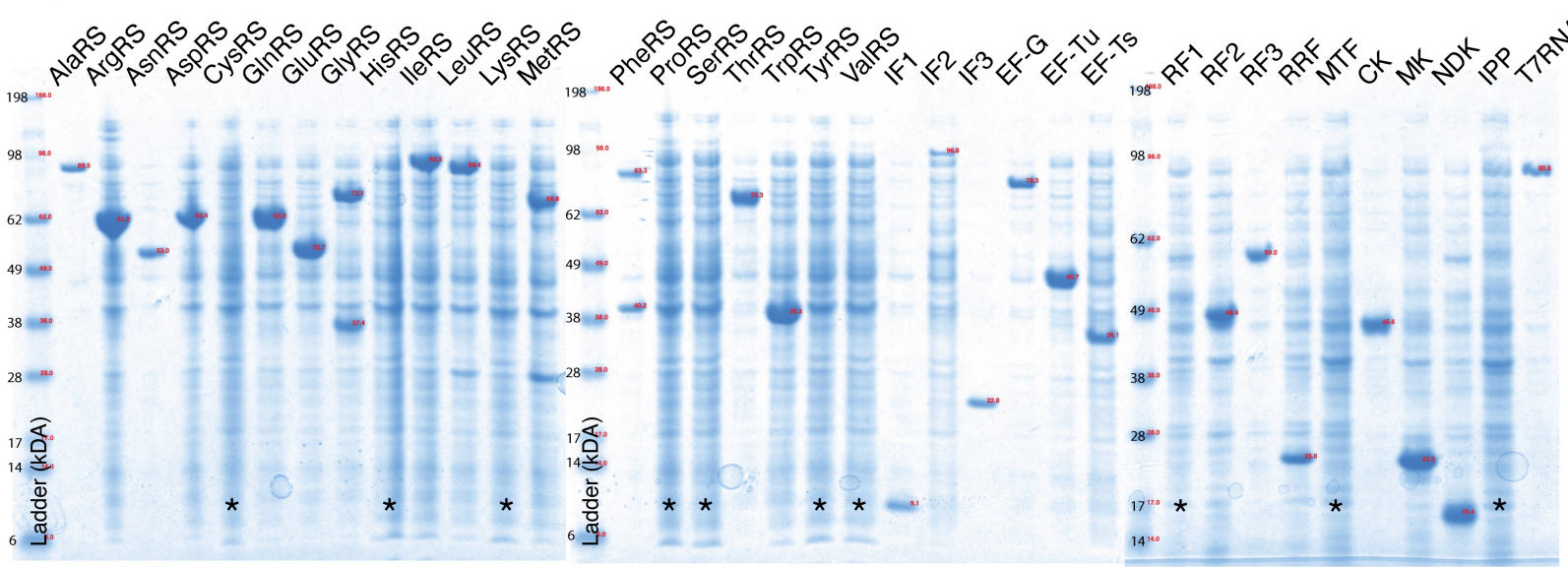
Grow cells in deep well plates



Induce protein expression w/ IPTG

Heat-denature cells to assess protein expression





SDS-PAGE gel revealed that **multiple PURE proteins** lost visible expression. This drop-out may be the cause of low productivity.

Unstable PURE protein expression is caused by background expression causing growth burdens

Host distribution of PURE protein expression

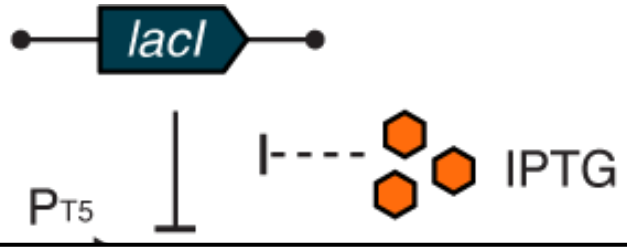
TX AARS TL En.Cyc

***E. coli* M15/pREP4**
16/36 plasmids

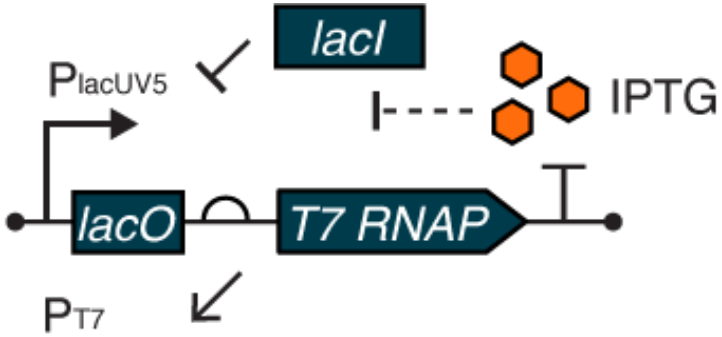
***E. coli* BL21(DE3)**
20/36 plasmids

Expression regulation mechanism

pREP4



λDE3



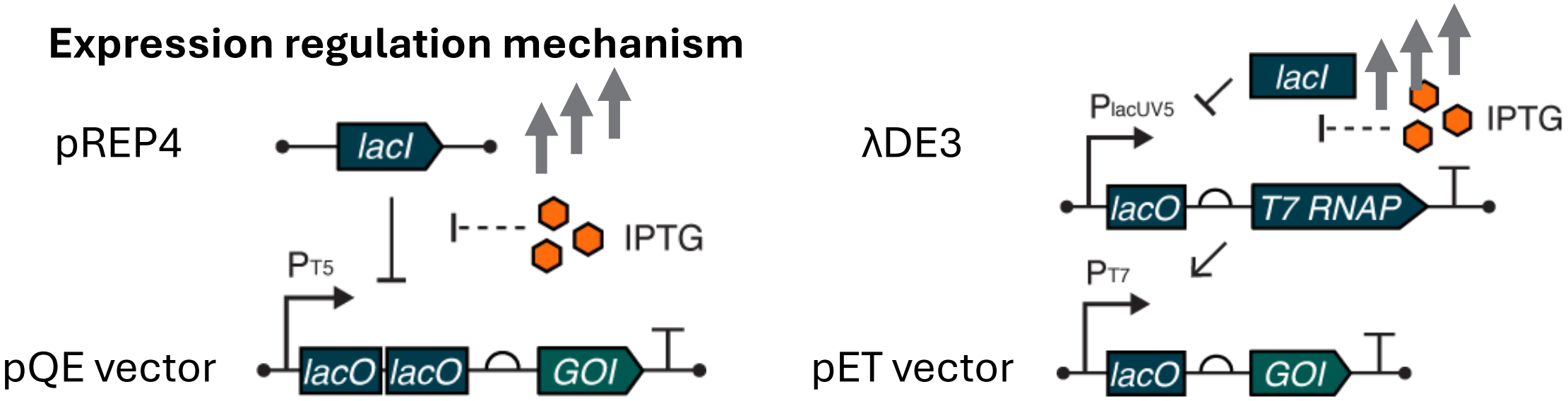
pQE

In both cases, insufficient LacI would lead to burdensome background expression of PURE proteins, contributing to expression instability

Adding glucose to overnight culture media improves expression stability via catabolite repression

Glucose-mediated catabolite repression
In the presence of glucose, cells will upregulate repressors to turn off other carbon utilization pathways.

Expression regulation mechanism



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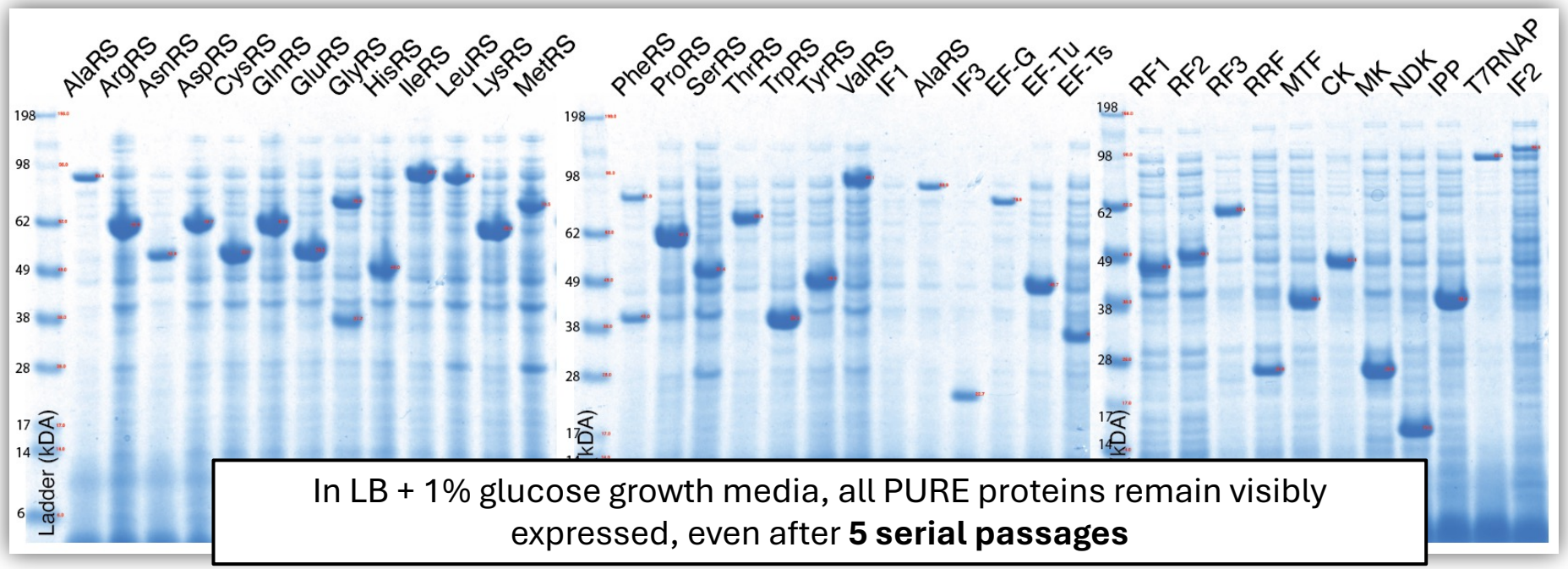
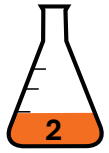


In LB + 1% glucose growth media, all PURE proteins remain visibly expressed, even after **5 serial passages**

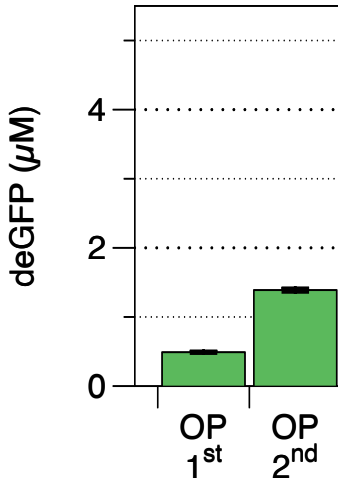
This is a common problem encountered by other labs.

Ngo *et al.* (2023) showed that expression stability can also be improved using BL21 Marionette strains carrying extra copies of LacI.

Suboptimal PURE protein stoichiometry also leads to an unproductive PURE system

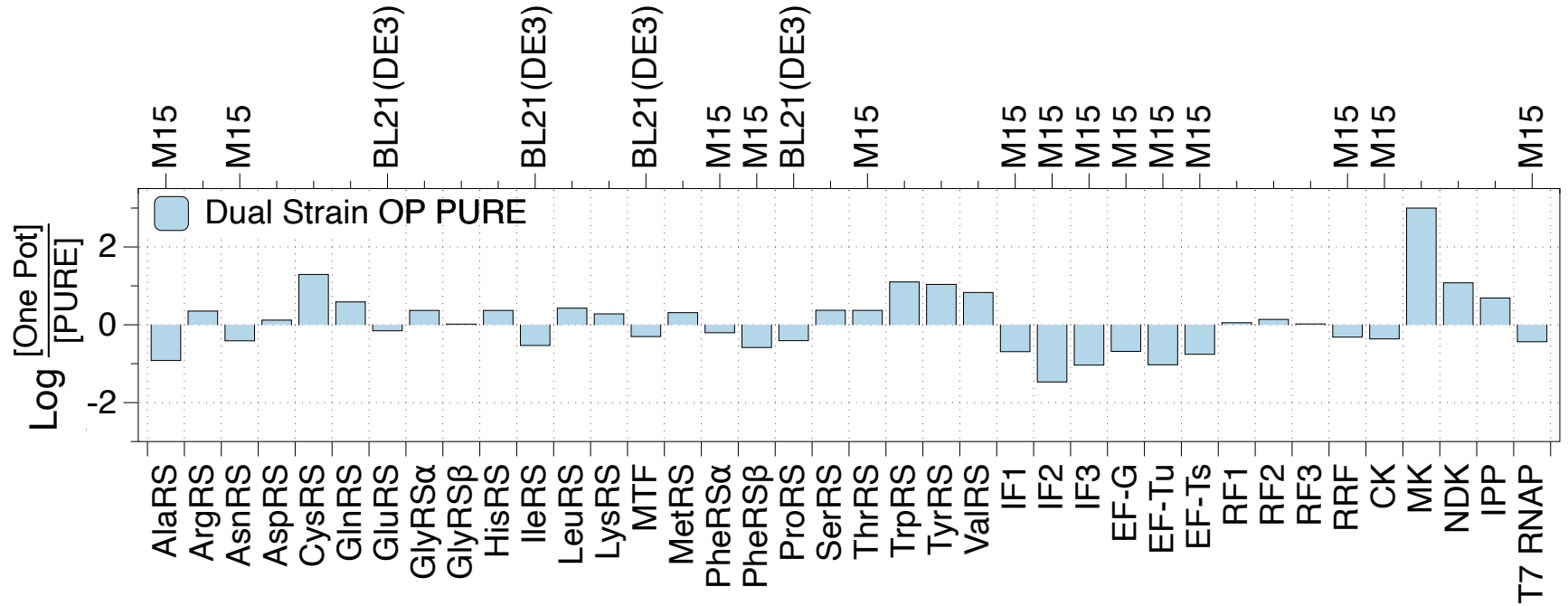


2nd attempt at One-Pot PURE



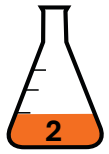
New batch is **3x more productive** than the previous batch.

But still **1/3 of the productivity** of commercial PURE



Mass spectrometry revealed the **abundance of many proteins in the One-Pot PURE mixture is lower** than that of commercial systems.

PURE protein deficiency may be correlated with slow *E. coli* M15 cell growth



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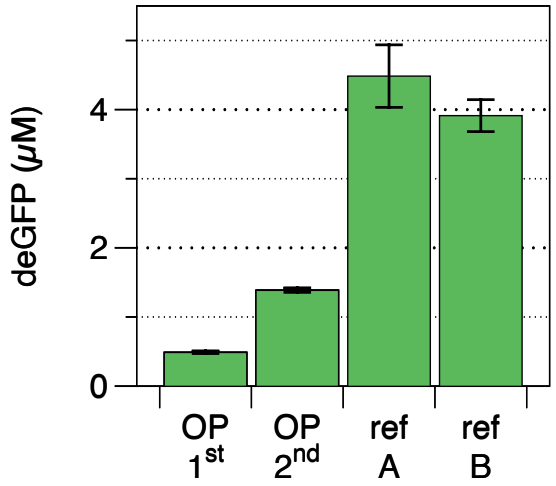

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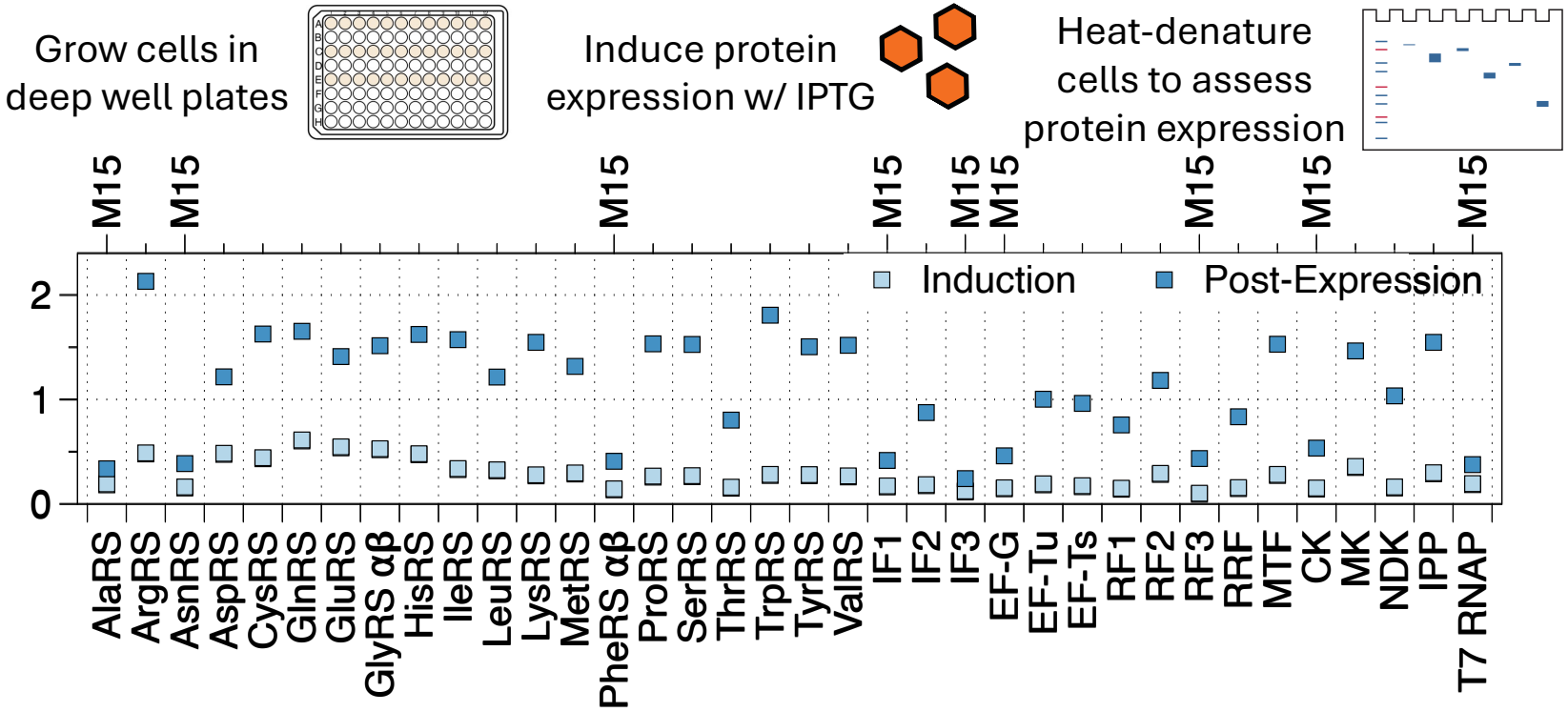
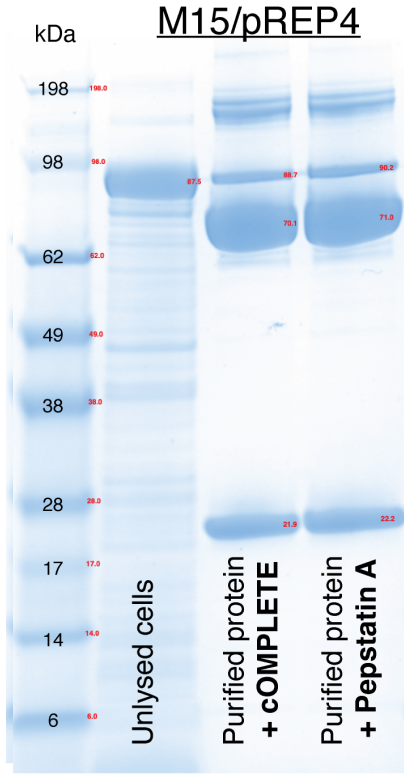


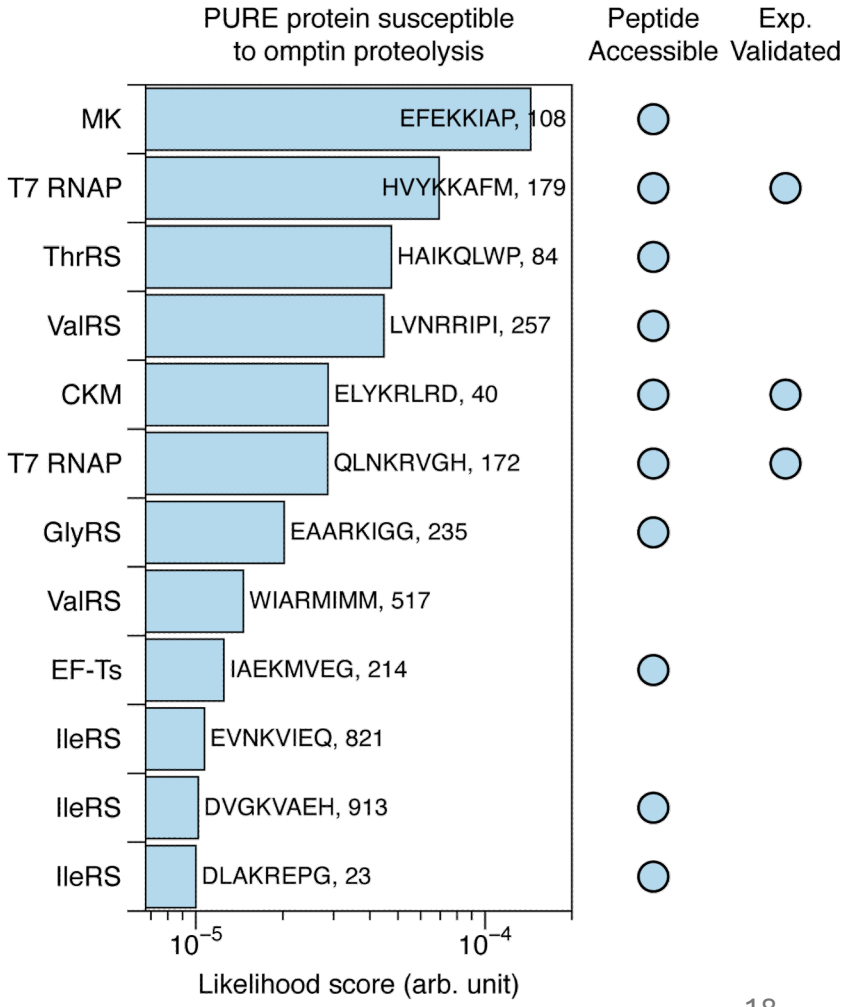
PURE proteins expressed in *E. coli* **M15 consistently grow more slowly**. The protein composition can be further reduced when grown in a co-culture.

E. coli M15 also exhibits omptin family proteolytic activities against PURE proteins

In purifying T7 RNAP from M15/pREP4, **proteolytic activity is observed at the purification** step, and this lysis is resistant to protease inhibitors.

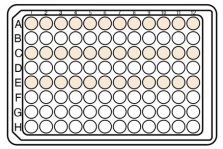


Based on OmpT's cleavage motifs, **at least 9 PURE proteins could be susceptible** to OmpT proteolysis



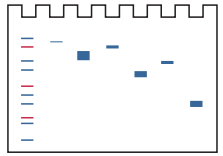
Expressing all PURE protein in BL21(DE3) led to more optimal protein stoichiometry and higher productivity

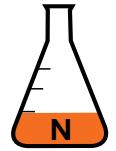
Grow cells in deep well plates



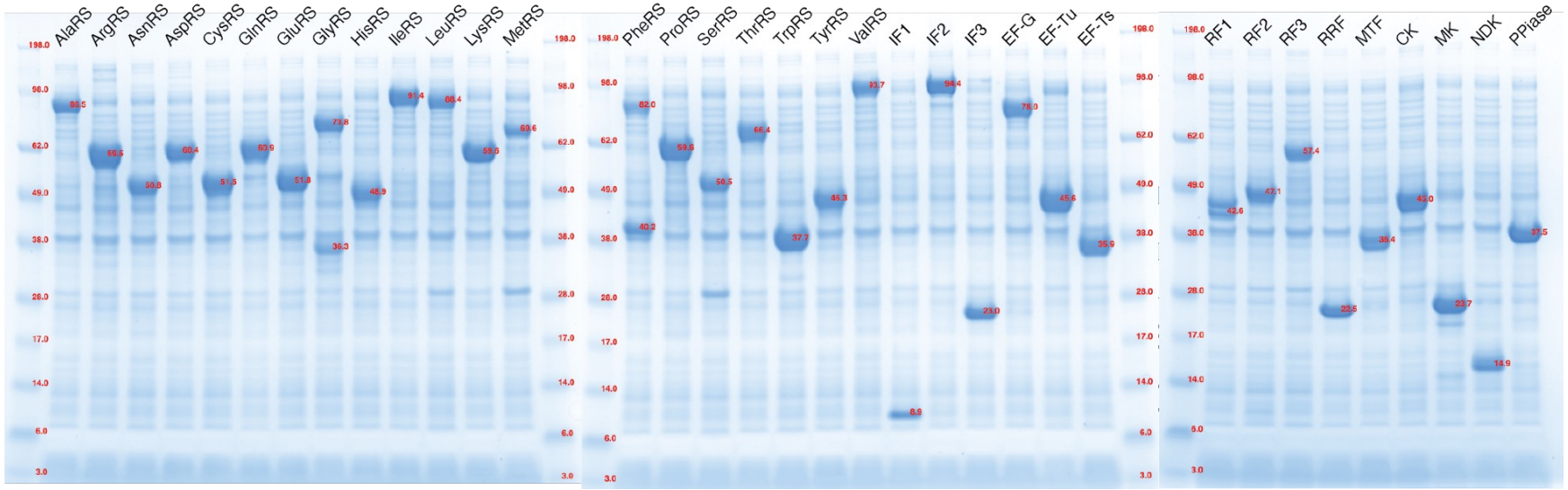
Induce protein expression w/ IPTG

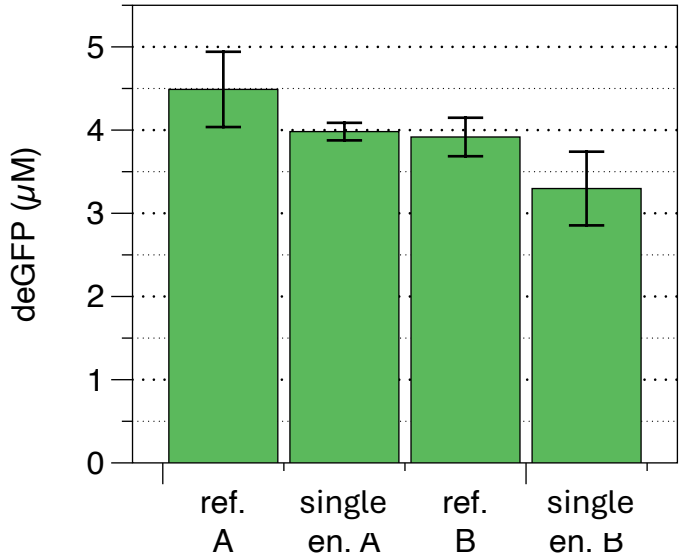
Assess protein expression





Last attempt at One-Pot PURE





*we note the original PURE system (Shimizu *et al.*, 2001) expressed all PURE proteins in BL21/pREP4.

The BL21(DE3) ver. of One-Pot PURE is **within 15% of the protein productivity** compared to commercial systems.

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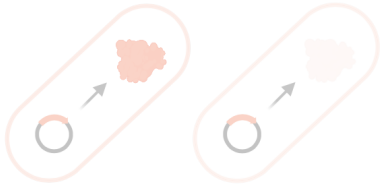
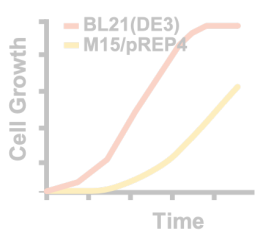
Unforeseen short-comings with the One-Pot PURE system and mitigating strategies

Loss of PURE protein expression



Slow growth and proteolysis in original expression strain



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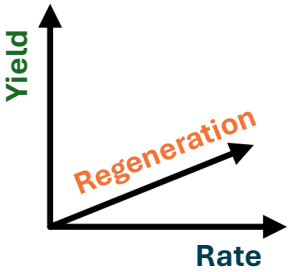
Some energy mixes are robust to a suboptimal PURE

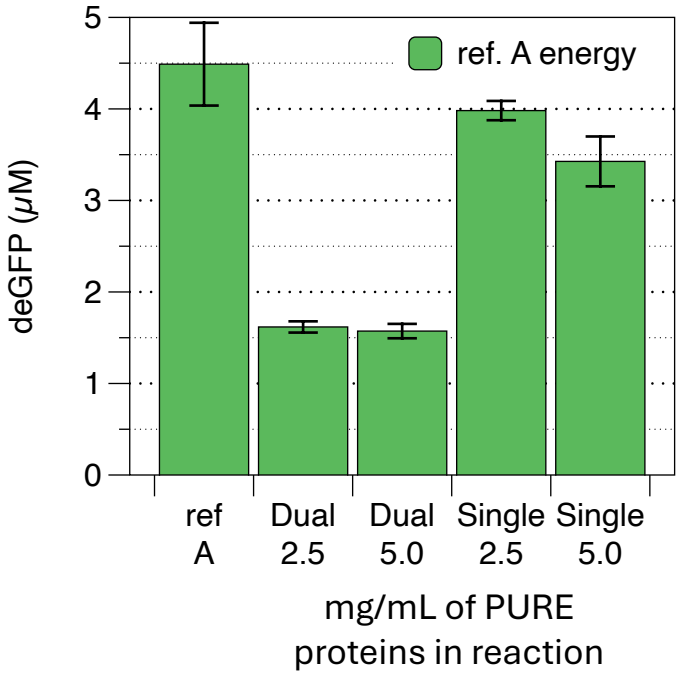
A balanced reaction rate is key to higher protein yield

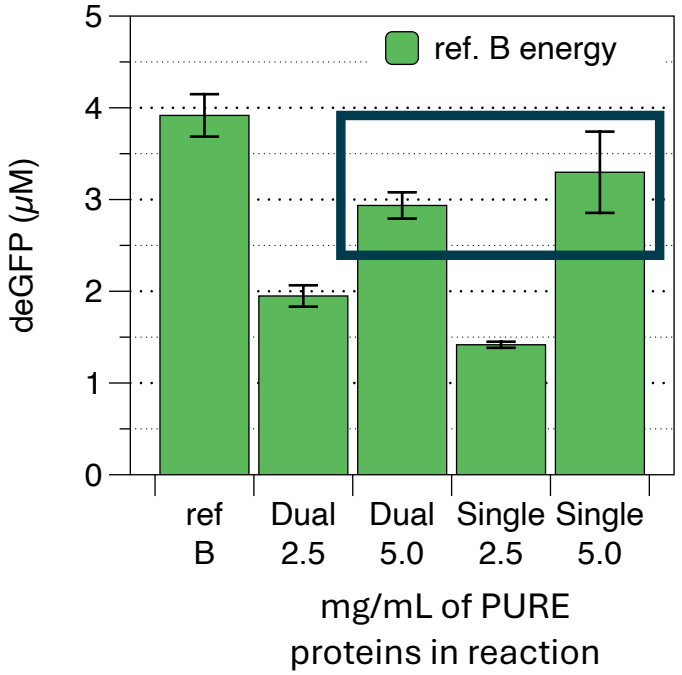


Biochemical composition can play a compensatory effect on sub-optimal PURE protein stoichiometry

Different One-Pot PURE system behaviors observed in two commercial energy mixes

Dual = One-Pot PURE made by M15 and BL21(DE3)
Single = One-Pot PURE made by BL21(DE3)





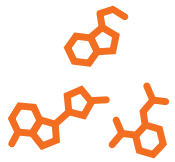
Vendor B's **energy mix is robust to a sub-optimal PURE stoichiometry**

Biochemical building blocks (NTP, AA, etc.) of energy solutions alone **cannot explain the drastic difference**

Transfer RNAs (tRNA) may be a source of under-appreciated complexity in the energy solution

tRNA composition alone strongly influences the protein expression rate and the final reaction yield

Prepared the PURE energy mix in-house



Original Formulation

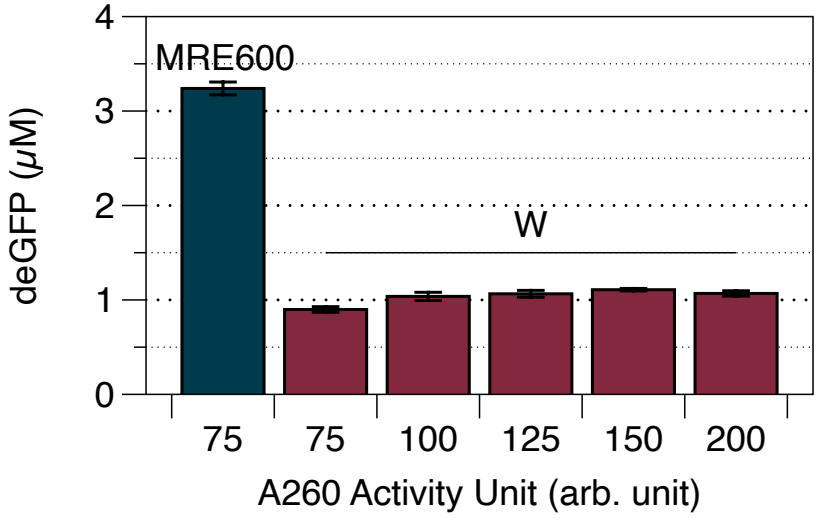
Varied the source of *E. coli* tRNA

MRE600 Strain W

Added in the PURE protein mixtures

Single Strain OP

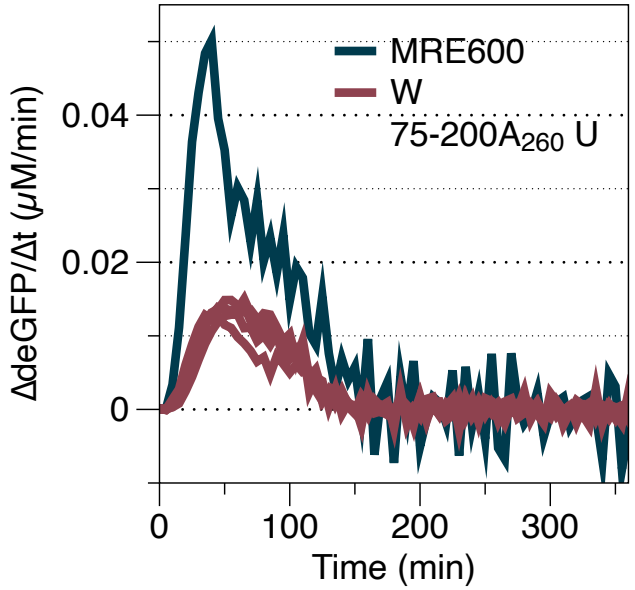
Comparing reaction yield



Reactions with **Strain W tRNA are 3-fold less productive** compared to MRE600 tRNA.

*We note that the *E. coli* MRE600 tRNA from Roche was discontinued in 2022.

Comparing translation rate

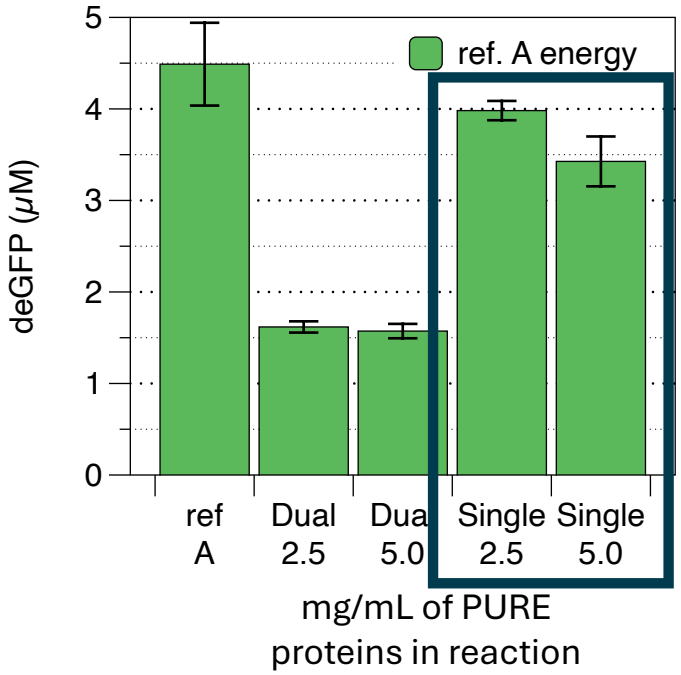


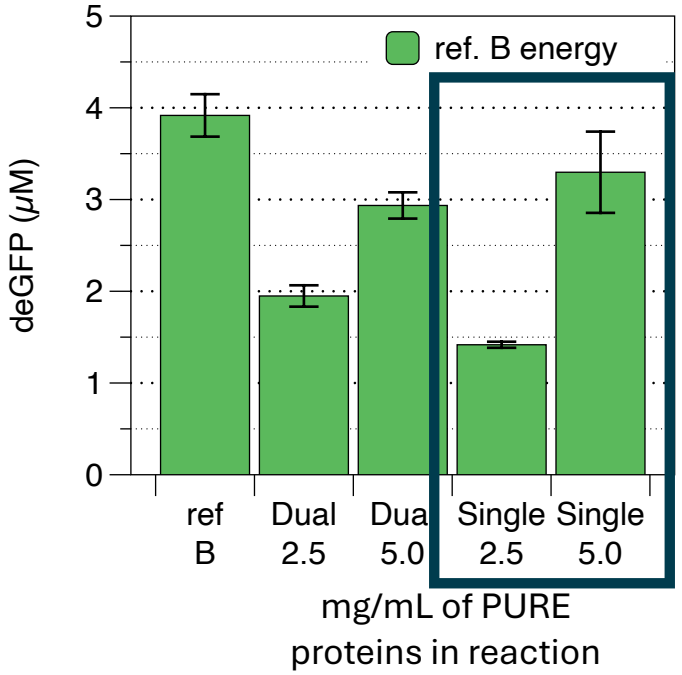
Strain W tRNA mix may have **deficient tRNAs, bottlenecking the reaction's translation capacity.**

High-yield protein expression requires a balanced protein production rate and energy formulation

Different One-Pot PURE system behaviors observed in two commercial energy mixes

Dual = One-Pot PURE made by M15 and BL21(DE3)
Single = One-Pot PURE made by BL21(DE3)



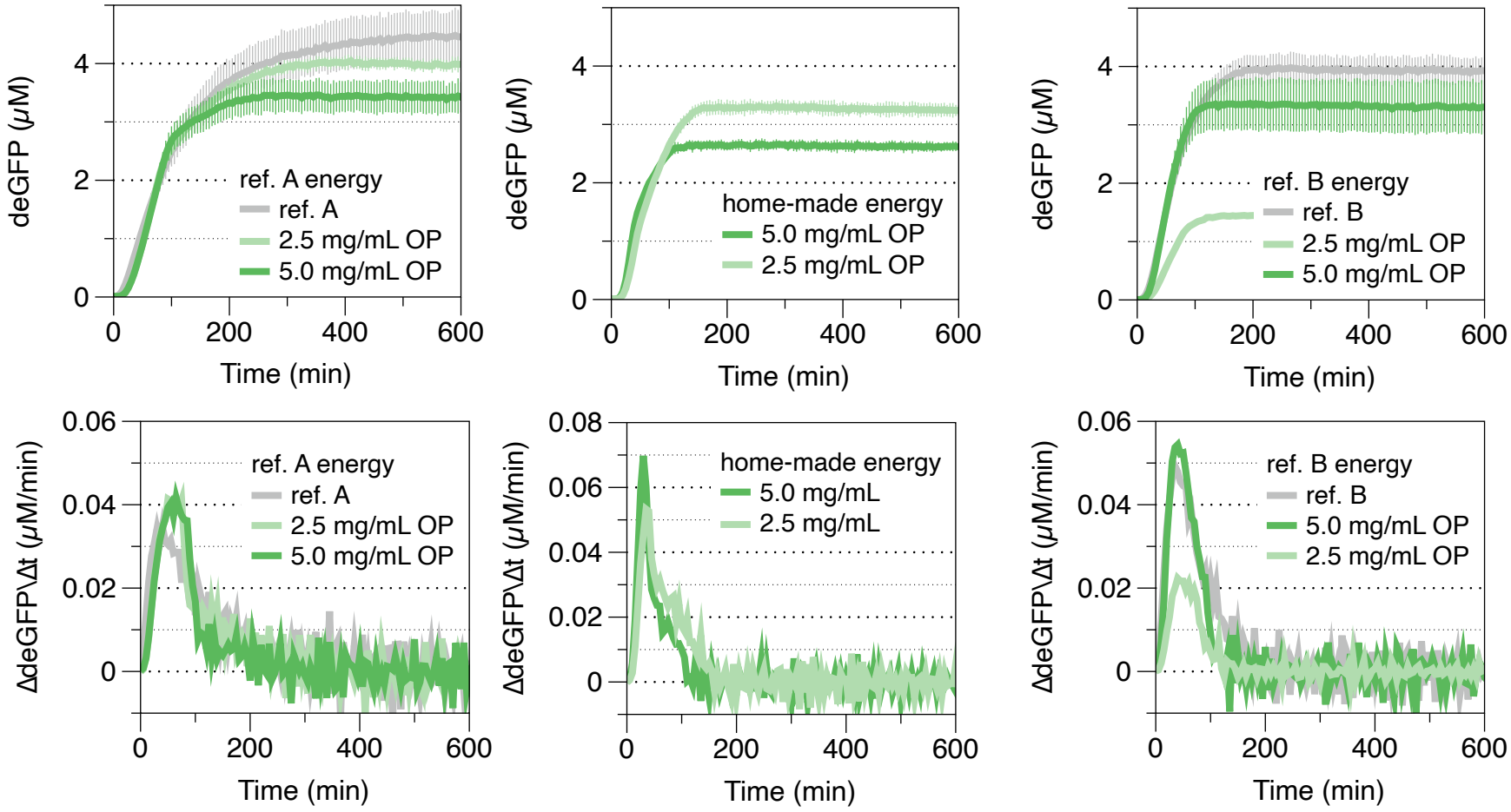


High-yield protein expression requires a balanced protein production rate and energy formulation

- Higher **$rate_{max}$ is correlated with higher rate-drop**
- Protein expression is energy-intensive. When the expression rate surpasses the system's energy regeneration rate, **ribosome stalling and premature termination** can happen
- But, the rate cannot be too low, where energy is spent without being productive

Too fast may be bad

Too slow can be worse



To set up a robust, productive One-Pot PURE

1. **Glucose-mediate catabolite repression** can be an effective strategy to improve genetic stability and prevent protein dropouts.

2. **E. coli M15 strain exhibits slower growth and proteolytic** activity, which is undesirable when making One-Pot PURE.

3. **The reaction's tRNA composition** strongly influences the protein expression capacity of PURE systems.

4. **High protein expression yield** in PURE systems requires balancing the expression and energy recycling rate.

Acknowledgment

Murray Lab Members

Collaborators




Prof. Paul Freemont and **Matas Deveikis** at Imperial College London

Prof. Tsui-Fen Chou and **Dr. Eric Qiu**, at Caltech Proteome Exploration Laboratory

Funding Sources

Resources

Slides for this talk

Manuscript preprint

Plasmids developed in this work are ready to distribute on Addgene