ENGINEERING CELL-FREE SYSTEMS FOR SYNTHETIC BIOLOGISTS

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Engineering Cell-Free Systems for Synthetic Biologists

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ABSTRACT

Synthetic biology (synbio) has emerged as a transformative scientific field with immense potential to address a wide-range of global problems. A specific sub-field of synbio utilizes cellular biomolecular machinery outside of a living cell. In theory, these “cell-free” systems offer a simpler approach and unique features compared to cell-based systems for biotechnology development. However, in practice limited accessibility and poor protein synthesis capacity hinder the overall scope and application of cell-free synbio. To address these challenges, it was our goal to create new engineering tools that will help expand the overall utility of cell-free expression systems. Data presented here provides: 1) detailed methods for the in-house preparation of a cost-effective *in vitro* reconstituted cell-free system, 2) an in-depth proteomic analysis of the system building blocks as a tool to characterize the composition and inform optimization, and 3) an improvement to protein synthesis capacity by modifying the ribosome composition. Furthermore, a critical assessment of the regulatory landscape is provided, promoting the safe and responsible use of cell-free synbio.

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LIST OF ABBREVIATIONS

aaRS	Aminoacyl-tRNA synthetase
ACN	Acetonitrile
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BIRRI	Biosecurity innovation and risk reduction initiative
BSA	Bovine serum albumin
CBD	Convention on biological diversity
CK	Creatine kinase
CRISPR	Clustered regularly interspaced short palindromic repeats
CTP	Cytosine triphosphate
DARPA	Defense Advanced Research Projects Agency
DBTL	Design-build-test-learn
DNA	Deoxyribonucleic acid
DHFR	Dihydrofolate reductase
DFHBI	3,5-difluoro-4-hydroxybenzylidene imidazolinone
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EF	Elongation factor
EIC	Extracted ion chromatogram
eGFP	Enhanced green fluorescent protein
ePURE	Ensemble protein synthesis using recombinant element
EYFP	Enhanced yellow fluorescent protein
FA	Formic acid
Fluc	Firefly luciferase
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GMO	Genetically modified organism
GMP	Guanosine monophosphate
GO	Gene ontology
GTP	Guanosine triphosphate
His	Histidine
iBAQ	intensity based absolute quantification
IDT	Integrated DNA Technologies
IF	Initiation factor
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	Luria Broth
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MAGE	Multiplex automated genome engineering
mRNA	messenger RNA or messenger ribonucleic acid
MK	Myokinase
MTF	Methionyl-tRNA formyltransferase
NDK	Nucleotide diphosphate kinase
NEB	New England Biolabs
NTPs	Nucleotide triphosphates
NTI:Bio	Nuclear threat initiative:Biosecurity division
Ni^{2+}NTA	Nickel Nitriloacetic Acid
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PURE	Protein synthesis using recombinant elements
PPiase	Inorganic pyrophosphatase
POI	Protein-of-interest
Rb	Ribosome
RBS	Ribosome binding site

RF	Release Factor
RNA	Ribonucleic acid
RRF	Ribosome release factor
TAK	Tris, ammonium chloride, and potassium
TL	Translation
tRNA	transfer RNA or transfer ribonucleic acid
TraMOS	Translation machinery one shot purification
TX	Transcription
T7 RNAP	T7 RNA Polymerase
UN	United Nations
UTP	Uridine triphosphate
UTR	Untranslated Region
SDS	Sodium dodecyl sulfate
sfGFP	Superfolder GFP

CHAPTER 1 - INTRODUCTION

1.1 PREFACE

My doctoral thesis focuses on a specific sub-field of synthetic biology (synbio), referred to as cell-free synbio. To set the stage for my thesis work and provide a frame of reference, Chapter 1 introduces cell-based and cell-free synthetic biology by briefly reviewing the history of the field and discussing recent advances and applications, with an emphasis on purified reconstituted cell-free expression systems. Current limitations are highlighted, building towards the purpose of my research goals. Lastly, I summarize the main objectives of my thesis work, and put into context the relevance of cell-free synbio and its benefits to society.

1.2 BRIEF INTRODUCTION TO THE FIELD OF SYNTHETIC BIOLOGY

Synbio is a diverse and dynamic field, constantly evolving as advancements are made. Broadly defined, it is the application of engineering principles to biology for the rational design and construction of new biological parts, devices, or systems or for the modification of existing living systems with practical utility (*1*). The enthusiasm surrounding synbio stems from the transformative potential of the associated biotechnology that could change how society addresses problems, offering sustainable and novel approaches to achieve global food and health security, clean energy production, and clean industrial manufacturing (*2*).

The key engineering principles applied to biology that have established the field of synbio include abstraction, decoupling, and standardization (*3, 4*). Due to the complexity of biological systems, abstraction and decoupling simplify how to solve a problem by focusing on a desired outcome, rather than understanding the specific details of how each component works (*3, 4*). Abstraction utilizes a system-oriented hierarchy to manage the intricate network of biological information, while decoupling provides the element of modularity or a way to deconvolute the design process (*3*). For example, a genetic circuit that performs a specific function is built from devices (e.g., small molecule sensor) that are made from genetic parts (e.g., promoters, ribosome binding sites (RBSs), and terminators) consisting of specific deoxyribonucleic acid (DNA)

sequences (AGTC) (*4*). Genetic circuits form modules with defined functions, which can then be combined in a plug-and-play manner for specific applications (Figure 1-1). By dividing the design process into simpler pieces (DNA→Parts→Circuits→Modules), more complex devices and systems can be put through the design-build-test-learn (DBTL) cycle enabling performance optimization using improvement to the constituent simpler devices, ultimately streamlining the construction of larger and more complex system(*1*).

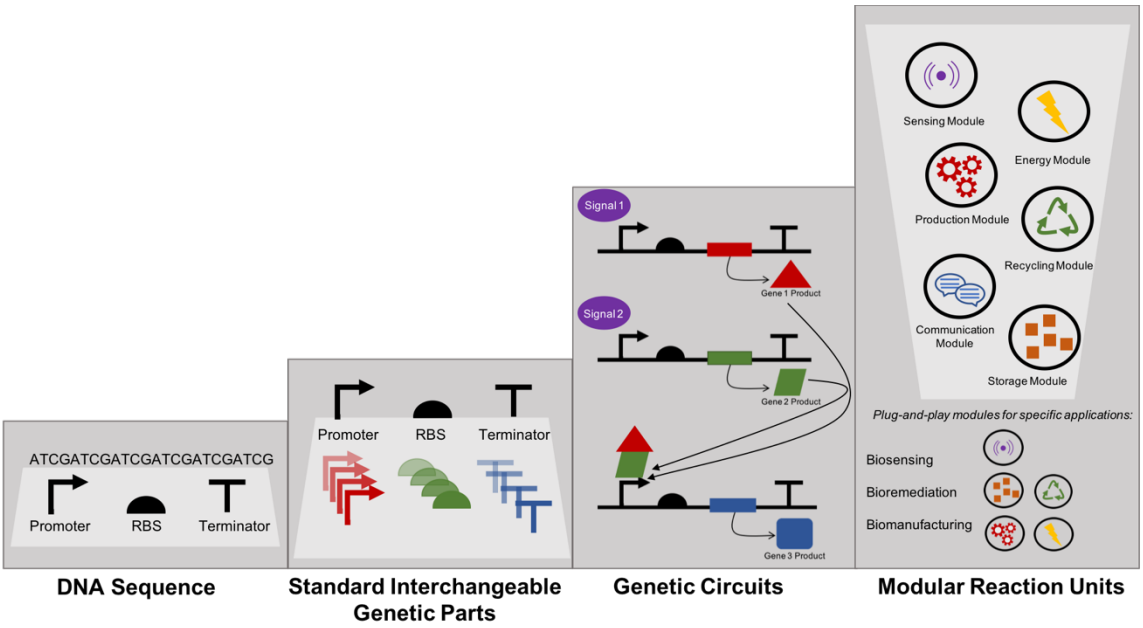


Figure 1-1: Application of the engineering principles abstraction and decoupling to biology result in a system-oriented hierarchy of biological components that can be combined to execute a user-defined function.

To build complex reaction pathways with a defined purpose, biological components are divided into simpler pieces; DNA sequences form the basic building blocks, which can be combined to create genetic parts with a specific function (e.g., transcription factor, terminator, RBS). The combination of genetic parts forms genetic circuits (e.g., logic gates, oscillators), which are combined to create modules that execute a higher level function (e.g., sensing, recycling, storage).

Accompanying abstraction and decoupling is standardization and interchangeability of these components for the reliable and predictable construction of bioengineered systems (*3, 4*). The goal for each hierarchal level is to include a library of components to choose from that have been tested and functionally characterized. As a result of standardization, well-characterized libraries of promoters, RBSs, and terminators are available (*5-8*). The BioBrick Foundation created the Registry of Standard Biological Parts, providing an open source repository of biological components, for researchers to use and contribute to (*9*). Synthetic genetic circuits have been designed analogous to electrical systems, including logic gates (*10-12*), switches (*13*), oscillators (*14*), and band-pass filters (*15*) enabling programming of specific biological functions and reaction networks. Progress in this area has been focused on addressing issues of context dependence, noise, and cross-reactivity to improve predictability and overall performance of the designed system (*16-18*). This also includes a better understanding of how each part performs in different chassis organisms.

Key scientific advances moving the field forward include an exponential decrease in the cost to synthesize and sequence DNA and an improvement in genetic engineering tools, such as CRISPR-based gene editing (*19*), allowing for cost-effective production and precise alteration of DNA (*20*). These enabling technologies lay the foundation for DNA to be managed and manipulated in a reliable and economical manner (*20*). Increasing computational power improves how to model and predict biological systems, including methods for rational design of novel proteins (*21*) and recoded genomes (*22*).

Biotechnology applications of synbio traditionally fall into two main categories: 1) a product produced by cells, or 2) engineered cells as a product themselves (e.g., a genetically modified organism (GMO)) (*23*). In the first category, examples include the synthesis of recombinant proteins, such as therapeutics like insulin to treat type 2 diabetes (*24*), the food additive heme to add the expected look and taste of meat to plant-based alternatives (*25*), and the synthesis of muscle fibres for applications in soft robotics (*26*). Engineered cells as a product include the modification of nitrogen fixing bacteria to be used as biological fertilizer, providing an alternative to chemical-based approaches (*27*), as well as the development of living therapies for the detection

of cancer in urine (*28*). In addition to engineered cells, the utilization of cellular biomolecular machinery outside the constraints of a living system (referred to as cell-free) have emerged as a sub-field within synbio, providing unique advantages and an alternative to cell-based engineering (*discussed in detail below*). Examples of cell-free biotechnology range from paper-based biosensors for the rapid detection of Zika virus (*29*) to the on-demand production of therapeutics, including those for the synthesis of COVID-19 antibodies (*30*).

Overall, a growing bio-economy is emerging (*31*). The commercialization of synbio has resulted in a multibillion dollar industry, advancing from a primarily research-based field to the development of commercially viable applications that positively impact society (*31*). By discovering new ways to better engineer biology and improve foundational aspects within the field, innovation and continued application is possible. Cell-free systems are an exciting piece to this next generation of synbio. For example, Debut Biotech, a cell-free biomanufacturing company, recently raised $22.6 million series A funding (*32*). Northwestern University in partnership with the U.S. Army announced the creation of a Cell-Free Biomanufacturing Institute to support the development of decentralized, on-demand manufacturing of useful products using cell-free systems (*33*). Furthermore, an article by Meyer and colleagues highlighted current trends within the field, including a 5-fold increase in the number of patent filings utilizing cell-free systems and an increase in the number of biotechnology companies incorporating cell-free synthetic biology (*34*). In general, cell-free approaches offer new and dynamic insights into the inner workings of cellular systems, and hold great potential for novel technological breakthrough with real world application.

1.3 HISTORY OF CELL-FREE SYSTEMS

The use of cell-free systems for biotechnology became apparent in the early 2000's (*35*). Prior to this, the machinery of cell-free systems was a valuable tool for the study of fundamental biological process, dating back to 1897 (*36*). An early example of this was demonstrated by the pioneering efforts of Nobel laureate Eduard Buchner (awarded the Nobel Prize in Chemistry in 1907) who discovered fermentation in yeast cell extracts (*36*). Almost 60 years later, cell-free systems were further developed as a research tool to understand gene expression. In 1954, Joane

Folkes and Ernest Gale studied the role of nucleic acids in protein synthesis using a model system that consisted of disrupted *Staphylococcus aureus* cells (*37*). Cell extracts isolated from rat liver cells led to the discovery of protein synthesis in microsomes and the requirement of adenosine triphosphate (ATP) and guanosine triphosphate (GTP) for this process (*38, 39*). In 1961, Nirenberg and Matthaei began to elucidate the genetic code, identifying amino acids encoded by nucleotide triplets in an *Escherichia coli* (*E. coli*) cell extract (*40*). These initial studies demonstrated the potential of cell-free systems, laying the foundation upon which current cell-free biotechnology has been built. Over the years, interest in cell-free systems has increased due to their broad utility and advantages over cell-based approaches (*discussed in Section 1.4*), in addition to their use for understanding fundamental biological processes.

1.4 CELL-FREE SYSTEMS AS AN ALTERNATIVE TO CELL-BASED SYNBIO

Synbio has traditionally relied on cellular hosts to execute genetic circuits, such as *E. coli* and *Saccharomyces cerevisiae* (*S. cerevisiae*), by hijacking the cell's natural capabilities for a user-designed purpose (*41, 42*). Cell-based approaches have numerous advantages for bioengineering (summarized in Table 1-1). However, cells are inherently complex, the components are difficult to standardize, and issues of part incompatibility and variability have limited their scope of applications (*43*). For example, reliance on the cell's biomolecular machinery leads to competition between the cell's natural metabolism and the bioengineered function, presenting a significant challenge in ensuring the system behaves as designed and to its optimal ability. The cell membrane acts as a barrier to the systems components, making it difficult to engineer, resulting in slow DBTL cycles (*44*). Controversy surrounding GMOs regarding their impact on the environment, as well as containment and regulatory concerns, has in some cases limited their widespread use.

Alternatively, cell-free systems have been explored by providing life-like functionality, including for the synthesis of ribonucleic acid (RNA) and protein, outside the constraints of living cells. These systems, which comprise cell lysates or are reconstituted from purified components (Figure 1-2), are of particular interest due to their relative ease of manipulation and direct control over system components (*45*). Desirable features include the ability to incorporate unnatural amino acids for the

production of proteins with novel functions, the synthesis of toxic compounds that are otherwise detrimental to living cells, a reduced biocontamination risk because components are unable to replicate or evolve, and increased freedom in physical properties; including protein activity and thermal stability (*35, 46*). Due to its open nature and lack of a cell membrane, cell-free systems enable rapid DBTL cycles and are useful for high throughput screening of genetic elements, (*refer to Section 1.7.2*). Additionally, linear DNA templates or direct messenger RNA (mRNA) can be used. Table 1-1 provides a comparison of both bioengineering approaches, highlighting key advantages of cell-free systems, as well as areas that require further improvement to match the performance levels of cell-based systems.

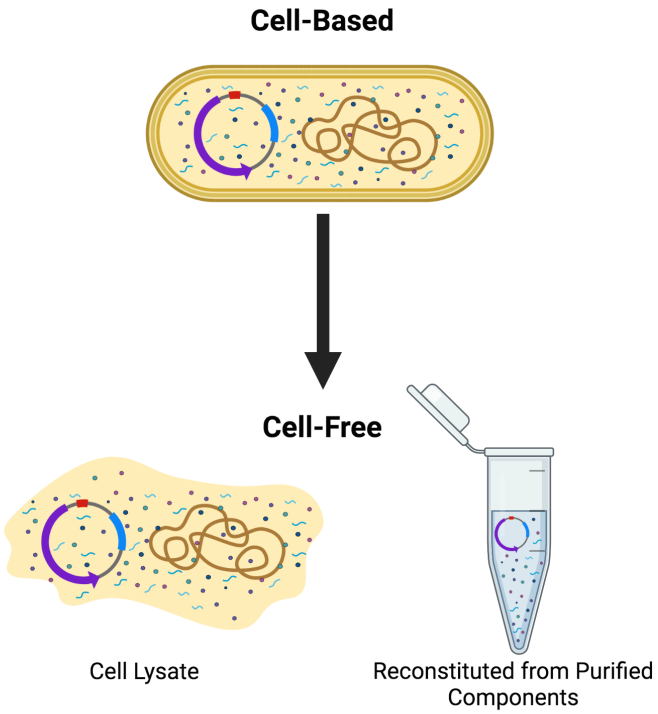
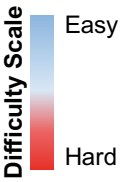


Figure 1-2: Cell-free systems contain the components required to execute life-like functions outside the constraints of a cell-based system.

Cell-free systems consist of cell lysates or are reconstituted from purified components that represent the minimal set of components required for gene expression. Image was created in BioRender.

Table 1-1: Design features of cell-based and cell-free bioengineering. Adapted from (*44*)

Feature	Cell-Based (*in vivo*)	Cell-Free (*in vitro*)
Ability to produce the desired compound	Difficult	Easy
Biocontamination risk	High	Low
Biomanufacturing ability	Requires cell lysis	Easy purification
	High production yield Simple scale-up	Low production yield Challenging to scale-up
Complex protein synthesis	Difficult	Easy
DBTL cycle	Slow	Quick
Incorporation of unnatural amino acids	Difficult	Easy
Control (e.g., transcription, translation)	Difficult (closed environment)	Easy (open environment)
Tolerance of Toxic compounds	Low	High
Engineering Tools Available	Many	Moderate
Post-translational modification	Easy	Difficult
Self-replication	Easy	Very difficult
Template	Plasmids, genomes	Plasmids, PCR products, mRNA



1.5 TYPES OF CELL-FREE PROTEIN SYNTHESIS SYSTEMS

Gene expression in a cell-free system is fueled by biomolecular machinery obtained from 1) crude cell extracts (also referred to as cell lysates) (*Section 1.5.1*) or from 2) highly purified, reconstituted transcription and translation (TX-TL) components (*Section 1.5.2*). In both cases, a protein-of-interest (POI) is produced following the addition of template DNA or mRNA. At the simplest level, cell-free systems can also be composed of individually purified enzymes for the *in vitro* reconstruction of biosynthetic reaction cascades. These types of cell-free systems have been developed for *in vitro* metabolic engineering and biomanufacturing applications, discussed in *Section 1.9.1*. Below, an introduction to the two main types of cell-free protein synthesis systems is provided, with an emphasis on optimizing performance and increasing accessibility of highly purified *in vitro* reconstituted TX-TL systems. An overview of bacterial translation is provided in Figure A1-1, as a reference.

1.5.1 CELL LYSATES

Crude cell extracts have been prepared from a diverse range of organisms including *E. coli*, *S. cerevisiae*, rabbit reticulocytes, wheat germ, insect cells, Chinese hamster ovary (CHO), and HeLa cells (*47-53*). A range of methods is available to prepare cell lysates, however each follow a relatively simple procedure consisting of a few basic steps that are similar across all cell

types. In brief, preparation includes cell culturing, cell lysis, and lysate clarification to isolate the TX-TL biomolecular machinery (*54*). This is followed by supplementation with components required for efficient gene expression such as nucleotide triphosphates (NTPs), transfer RNA (tRNA), amino acids, salts (e.g., magnesium or potassium), and energy rich compounds (e.g., phosphoenolpyruvate or creatine phosphate) (*54*). Although preparation of a cell lysate is considered simple and inexpensive, batch-to-batch variation is common and the composition of the system is relatively unknown and difficult to control, with the potential for the presence of unwanted DNase and RNases (*55*). Therefore, optimization is required for consistant preparation, as well as approaches to improve protein yield and overall system performance.

Bacteria-based cell extracts, specifically *E. coli*, are the most common and well-studied cell-free protein synthesis systems. Streamlined and high-throughput methods have been described for the isolation of an *E. coli* cell lysate by centrifugation at 30 000 xg (referred to as a crude S30 extract) (*56, 57*). Exploring different cell growth conditions for isolation, such as heat stressed non-growing *E. coli,* resulted in cell lysate that was less labour intensive to produce generating higher extract yields, comparable to traditional cell lysate protein synthesis activity (*58*). Additionally, by growing cells under stress, the protein composition can be modified resulting in expression of alternative transcription factors (*58*). Small changes in cell growth conditions can have a large effect on the overall cell lysate composition, contributing to batch-to-batch variability.

In recent years, the *E. coli* cell lysate has become less of a “black box”, with the identification of 821 core proteins, elucidated through proteomic analysis (*59-61*). As a result of such work, an overview of proteins that are beneficial or potentially harmful to gene expression was provided. To modify the protein composition and inactivate the genes that have a negative effect on *in vitro* protein synthesis (such as nucleases or proteases), multiplex automated genome engineering (MAGE) has been employed, resulting in the creation of high performance strains (*62*). Furthermore, strain engineering has been used to genomically recode all UAG codons to UAA freeing the UAG codon for non-canonical amino acid incorporation (*63*). Strains have also been created that encode for T7 RNA polymerase (T7 RNAP), enabling orthogonal expression of genes under the control of a T7 promoter (*63*). Outside of strain engineering, “toolboxes” have been

developed to optimize system properties, including the addition of a MazF interferase to adjust mRNA levels, and the protease ClpXP AAA+, to control protein degradation (*64*). Modifying the genome to express a recombinant protein or supress an endogenous factor, as well as supplementing the cell lysate with purified components, provides two approaches to control the lysate composition.

To obtain other desirable features for gene expression and take advantage of natural cellular process that do not exist in *E. coli*, different types of cell extracts are used. For example, eukaryotic-based extracts are a preferred option for glycosylation, post-translational modifications, and disulfide bond formation, which are important features for the function of some therapeutic proteins, such as antibodies (*65*). Cell extracts isolated from thermophilic organisms allow for the expression of recombinant proteins at high temperature (*66*). Fast growing organisms with short doubling times and high protein synthesis levels such as *Vibrio natriegens*, are desirable options for efficient extract preparation resulting in high protein yield (*67*). Overall, each type of cell extract provides different characteristics that impact gene expression. Some are well-characterized and easy to prepare, while others are more challenging but provide unique features for recombinant protein synthesis (*54*).

1.5.2 PURIFIED RECONSTITUTED

The first cell-free system composed of individually purified components was reported in 2001 by Shimizu *et al.* (2001) referred to as the protein synthesis using recombinant elements (PURE) system (*68*). It was composed of the minimal components required for TX-TL, including initiation factors (IFs: IF1, IF2, IF3), elongation factors (EFs: EF-Tu, EF-Ts, EF-G), release factors (RFs: RF1 and RF3), a ribosome recycling factor (RRF), 20 aminoacyl-tRNA synthetases (aaRS's), methionyl tRNA transformylase (MTF), and a transcription factor (T7 RNA polymerase). The 32 proteins were polyhistidine (His)-tagged and individually purified by nickel affinity chromatography, while purified ribosomes were isolated by sucrose-density-gradient centrifugation. Additional factors, including creatine kinase (CK), myokinase (MK), and nucleoside-diphosphate kinase (NDK), required for energy regeneration, as well as inorganic pyrophosphatase (PPiase), tRNAs,

amino acids, NTPs, and creatine phosphate, were purchased commercially and added to the system. Table A1-1 summarizes the functional role of each component for *in vitro* protein synthesis.

The composition of the PURE system is defined, therefore it is theoretically considered free of unwanted factors that might interfere with the designed function, such as nucleases and proteases. By reconstituting the system from purified components, the composition of the system can easily be modified, providing features of improved engineerability and control. To achieve the necessary high purity, time-intensive and laborious preparation is required, providing a potential drawback in comparison to producing a cell extract in-house. Alternatively, the PURE system can be purchased commercially, for example from New England Biolabs (NEB) as the PURExpress® Kit and from GeneFrontier Corporation as the PUREfrex® system. The PUREfrex® system is similar to PURExpress®, except the protein component does not contain any histidine affinity tags. Although readily available, commercial systems are expensive, hindering the scale of reactions that can be performed depending on monetary constraints. Furthermore, the system modularity or customization is limited; only three alternative PURExpress® kits are available, separating the RFs, ribosomes, or tRNA and amino acids from the complete mixture (PURExpress® Δ RF123, PURExpress® Δaa/tRNA, PURExpress® ΔRibosomes).

In an aim to increase accessibility, methods have been developed to simplify the purification process and provide cost-effective alternatives to the commercial PURE systems (Table 1-2). The first approach used MAGE to insert his-tag sequences directly into the *E. coli* genome for co-purification of the 36 translation machinery components from 6 strains (including elongation factor-4 (EF-4)) (*69*). The resulting ensemble PURE system (ePURE) was less active than the commercial system, demonstrating 11% of the protein synthesis activity for the expression of dihydrofolate reductase (DHFR). Shepherd *et al.* (2017) constructed three rationally-designed plasmids expressing 30 of the 31 translation factors, each encoded in BioBrick standard and his-tagged for purification by nickel affinity chromatography (*70*). Three expression and purification steps were required to isolate each translation factor for the reconstituted system, termed PURE 3.0. This is significantly reduced from the 31 individual expressions and purifications that would normally be required. Proteins were grouped based on the amount needed in the final system,

resulting in a low, intermediate, and high classification. EF-Tu was not included due to the much higher levels required for efficient protein synthesis and was purified separately. Following purification and reconstitution with ribosomes, substrates, and cofactors, similar levels of protein synthesis were observed for PURE 3.0 compared to the PURExpress® system. To further simplify this process, all 30 factors were ligated into a bacterial artificial chromosome. Although construction of the ~65kb plasmid was successful as verified by real-time single molecule sequencing, further optimization was required to produce a functional *in vitro* translation system.

Rather than constructing plasmids encoding polycistronic mRNA for the expression and purification of multiple proteins at once, Villarreal *et al.* (2018) explored the benefit of microbial consortium, where multiple cell strains were each engineered to express a different translation factor and cultured together. Through density-controlled co-culture, recombinant proteins were purified from multiple cell strains in a single cell lysis and purification step (*71*). This method was referred to as the translation machinery one shot (TraMOS) purification, and was applied to the purification of 34 factors required for *in vitro* translation (including EF-4). Rationally designed RBSs and plasmid copy number allowed for fine-tuning of the protein expression levels. Protein synthesis activity was within the range of reported PURExpress® yields, but when directly compared to the yield of green fluorescent protein (GFP) resulted in ~20% of the protein synthesis activity. Additionally, TraMOS was considered cheaper to produce, reporting 0.18 USD per µL compared to 1.36 USD per µL for the PURExpress® system.

A similar approach described by Lavickova *et al.* (2019) was referred to as the One-Pot purification method, where a single cell-lysis and purification step was employed following the co-culture of 36 *E. coli* strains, each expressing a component of the translation machinery, including energy regeneration components (*72*). Equal volumes of inoculating culture were combined for each of the 36 strains. Increasing the percentage of EF-Tu in the co-culture to 47% resulted in an *in vitro* translation system capable of similar enhanced GFP (eGFP) yields in comparison to a PURE system. In addition to the translation factors, methods were provided to produce ribosomes and an energy solution in-house. A detailed cost analysis revealed a significant decrease in cost to produce

the OnePot system (0.09 USD per µL), providing an inexpensive alternative to the standard PURE system while providing similar functionality.

Table 1-2: Comparison of purified, reconstituted *in vitro* TX-TL systems

System	Reported Yield (µg/mL)	Cost Analysis* (USD/µL)	Features
PURE (*68*)	10-200	1.36**	-individual expression and purification -31 translation factors (minus RF2, CK, MK, NDK, PPiase)
ePURE (*69*)	22	N/A	-Genomic insertion of his-tags -36 translation factors (including EF-4, minus T7 RNAP)
PURE 3.0 (*70*)	150-300	0.033***	-Constructed three expression plasmids -3 protein expression and purification steps -30 translation factors (minus EF-Tu, T7 RNAP, CK, MK, NDK, PPiase)
TraMOS (*71*)	31.7	0.18	-engineered synthetic microbial consortia -single culturing, lysis, and purification -32 translation factors (included EF-4, minus T7 RNAP, CK, MK, NDK, PPiase)
OnePot (*72*)	156	0.09	-co-culture of expression strains -single expression and purification step -36 translation factors

*different factors were considered for each cost analysis, and price of components may vary between years, impacting a direct comparison between systems.
**commercial cost of the PURExpress® in 2021
*** not including labour, equipment, or overhead

In addition to increasing the accessibility of *in vitro* reconstituted cell-free systems, methods to improve functionality and protein yield have been explored, including fine-tuning the concentration of specific components and/or supplementing the reaction with additional factors. For example, EF-Tu concentration is critical. EF-Tu is the most abundant protein in *E. coli* with a cellular concentration approximately 10 times the amount of ribosomes during rapid growth (*73*). Supplementing a PURE reaction with additional EF-Tu has increased protein synthesis activity (*72, 74*). Li *et al.* (2014) demonstrated the benefit of optimizing the concentration of EFs, RFs, RRF, and tRNA (*74*). Additionally, EF-4 (which has been included in ePURE and TraMOS), chaperones GroEL/ES, and the crowding agent bovine serum albumin (BSA) were added resulting in an increase in protein yield by ~5-fold (in the case of the reporter protein, firefly luciferase (Fluc)) (*74*).

Critical concentrations of other translation factors and components of the cell-free expression systems have also been identified. For example, increasing the RF and RRF concentrations from 2 µM to 5 µM each, or the IF concentrations from 2 µM to 4 µM each,

significantly reduced Fluc fluorescence, demonstrating an optimal concentration for gene expression (*74*). Regarding cofactors, magnesium ions (Mg^{2+}) are essential for a variety of biological processes, most notably protein synthesis playing a critical role in ribosome assembly and for the activity of Mg^{2+} dependent enzymes (*75*). A ~50% increase in protein yield has been observed by feeding magnesium to offset sequestration by inorganic phosphate (*76*).

Kazuta *et al.* (2008) conducted a high throughput analysis to assess the effect of individual *E. coli* proteins on the *in vitro* expression of GFP. Of the 4194 proteins tested, 12% had an effect on protein expression and 34% of these factors were directly linked to translation machinery (*77*). A portion of these factors were found to have a positive effect on protein synthesis (e.g., RNA helicase, HrpA; oligoribonuclease, Orn; thioredoxin 2, TrxC; chaperones Tig and SlyD; carbon-phosphorus lyase complex subunit, PhnH), while others had a negative effect (e.g., transcriptional repressor LacI; toxins ChpA, ChpB, RelE, YoeB; nuclease Rnt; uncharacterized protein YhaV; DNA polymerase I, PolA; GTP cyclohydrolase II, RibA). Overall, this work demonstrates the complex functional network and challenges associated with reconstituting a minimal *in vitro* translation system that is highly functional and accurately mimics the *in vivo* processes.

Other ways to improve the PURE system include alternative approaches to regenerate energy. In the PURE system, NTP regeneration is achieved by three kinases; CK transfers the phospho-moiety of the substrate creatine phosphate to adenosine diphosphate (ADP), MK and NDK consume ATP and transfer a phospho-moiety to adenosine monophosphate (AMP) and guanosine diphosphate (GDP), respectively. This approach depletes the ATP pool for the regeneration of ADP and GTP, and also requires an expensive substrate. To simplify and improve the energy regeneration system, Wang *et al.* (2020) replaced the three kinases with a single bi-functional polyphosphate kinase (*78*). The class III polyphosphate kinase (CHU0107t) from *Cytophaga hutchinsonii* can phosphorylate AMP, ADP, guanosine monophosphate (GMP), and GDP by transferring the phospho-moiety of inorganic polyphosphate (polyP), which is an inexpensive substrate compared to creatine phosphate. The single-kinase system was capable of driving protein synthesis, resulting in superfolder GFP (sfGFP) yields of ~530 µg/ml compared to 400 µg/ml for the three-kinase system.

In the above mentioned systems, the required tRNAs were purchased commercially. Alternatively, Hibi *et al.* (2020) reported the addition of *in vitro* transcribed (IVT) tRNA, resulting in 40% of the protein synthesis activity compared to the PURE system for the expression of DHFR (*79*). Although a lower yield was observed, it is important to note that IVT tRNA lack any nucleotide modifications (*80*). By preparing the tRNA in-house, unique features for genetic recoding and potential system improvements are available. However, lower protein yields requires further improvement.

A common conclusion reported by many studies is the observation of poor protein synthesis resulting from inefficient ribosomes or ribosome-associated processes (*76, 81-83*). Kempf *et al.* (2017) aimed to elucidate what aspect of ribosome performance impacts translation by investigating the fraction of actively translating ribosomes compared to the number of translation cycles. Their results estimated that only approximately 40% of ribosomes initiate translation, followed by only two rounds of translation (*83*). Through atomic force microscopy, Doerr *et al.* (2019) similarly identified poor translation initiation with only 10% of ribosomes actively translating, which, in both cases, is significantly lower than the ~80% of ribosomes initiating translation *in vivo* (*81*). The presence of truncated products has also been identified by mass spectrometry and linked to “impaired ribosome processivity”, which may be caused by ribosome stalling, premature ribosome termination, or truncated mRNA (*82*). Ribosomes, as well as tRNA, represent a relatively unexplored area regarding the identification of alternative purification strategies and optimized functionality in comparison to the protein component of the PURE system. Therefore, future work needs to address these issues to reach optimal protein synthesis capacity *in vitro.*

1.6 DEPLOYMENT STRATEGIES

There are a variety of ways to execute a cell-free system, including batch, continuous exchange or continuous flow reaction formats, containment within a capsule, embedded within a hydrogel, or lyophilized on paper. The physicochemical properties and functionalities of each are important considerations when deciding on how to execute a cell-free reaction, and are summarized below.

1.6.1 BATCH, CONTINUOUS EXHANGE, AND CONTINUOUS FLOW REACTIONS

Batch mode provides the most simplistic or basic approach to execute a cell-free reaction. All of the components are combined in a single vessel, such as a test tube or 96-well plate, and are incubated with a template to produce the POI

. Batch reactions are suitable for proteins with high expression levels, as well as for high throughput screening or initial tests due to the simple nature of the reaction set-up and minimal components required (*54*). On the other hand, batch mode is limited by the amount of substrate or energy available (e.g., the PURE system reaches maximum yield at ~4 hours), the impact of toxic or inhibitory by-products (e.g., free phosphate), and the cost of scale-up in regards to the more expensive purified reconstituted systems (*84*).

To increase the duration of the reaction and improve titers, continuous exchange and continuous flow reactions have been employed. For continuous exchange, the reaction is separated from a feed-stock by a semi-permeable dialysis membrane that allows for substrates and energy rich molecules to diffuse into the system while allowing toxic by-products to flow out (*85*). Similarly, continuous flow reactions supply a constant flow of energy rich feed-stock and exhibits passive diffusion through an ultrafiltration membrane to separate out the POI and other by-products (*86*). For more automated, high throughput analysis, microfluidic technology has been used, allowing for precise manipulation of geometrically constrained fluids of a small reaction scale (*87*).

1.6.2 ENCAPSULATION

Encapsulation of cell-free systems provides a way to mimic the cellular membrane, creating a crowded environment similar to *in vivo* conditions, enabling the development of synthetic cells and origins-of-life research while also maintaining reduced complexity and providing control over the membrane composition (*88, 89*). In addition to this purpose, encapsulation provides features of spatial and temporal control, the ability to regulate metabolite flux, reduced environmental interference, and compartmentalization of specific reaction components (*90-92*). These features are highly amenable for biotechnology development and have most commonly been explored as vehicles for drug delivery (*93*). A variety of membrane-bound compartments have been developed,

including lipid-based (liposomes) (*94*), protein-based (proteinosomes) (*95*), or polymer-based (polymersomes) (*96*) capsules, as well as the formation of hybrid systems (*97*).

Liposomes represent the most common compartment for encapsulating a cell-free system and are composed of one or more self-assembling phospholipid bilayers to form a closed, spherical vesicle. There are many different types of liposomes characterized by the composition, vesicle size, and method of preparation (*98*). Two common approaches to prepare liposomes are the 1) thin film hydration method, where a dried lipid film is hydrated with a cell-free reaction mixture (or solution to be encapsulated) resulting in lipid swelling and subsequent multilamellar vesicle formation (*99*) and 2) emulsion-based techniques, where lipids are dissolved in an organic solvent and mixed with the solution to be encapsulated, resulting in the formation of a lipid monolayer, followed by solvent evaporation (*100*). Low encapsulation efficiency and vesicle polydispersity are common issues (*101*). Droplet-based microfluidics have improved on this by enabling the rapid and reproducible formation of monodisperse liposomes through optimized fluid control (*102*).

Encapsulation of cell-free biomolecular machinery produces efficient small-scale bioreactors for protein synthesis by providing an answer to the dilution problem experienced in bulk conditions (*103, 104*). In addition to compartmentalized gene expression, other biological reactions have successfully been executed within a vesicle, including DNA replication, polymerase-chain-reaction (PCR), reverse transcription (RT)-PCR, and a variety of enzymatic pathways (*105-107*). Liposomes have also been used as a tool for the *in situ* synthesis of membrane proteins, leading to membrane integration and the formation of proteo-liposomes (*refer to Section 1.7.1*). Along with providing a biomimetic platform for the study of cellular components, proteo-liposomes facilitate exchanges between the internal and external environment, a desirable feature for the construction of synthetic signal transduction pathways. The membrane protein, alpha hemolysin, has commonly been exploited for this purpose, enabling communication between different populations of liposomes for the execution of a reaction cascade (*108, 109*). Overall, liposomes are a useful tool for the synthesis of difficult-to-express proteins (*expanded on in Section 1.7.1*), as well as for applications in directed evolution (*expanded on in Section 1.7.3*).

Encapsulation efficiency, membrane composition, biocompatibility, vesicle size, vesicle uniformity, membrane stability, and the potential for programmed functionality are all important properties to consider when deciding on the type of capsule to be used. Liposomes are typically inert, conveying no additional capabilities. The incorporation of membrane proteins improves on this lack of functionality, however, it is difficult to ensure correct protein orientation and precise integration into the membrane (*110*). Alternatively, protein-based capsules, such as bacterial microcompartments and proteinosomes, form a biocompatible and enzymatically active membrane with the ability to self-assemble and selectively alter permeability (*97, 111*). Wang *et al.* (2019) drew inspiration from jellyfish to construct a "breathable" proteinosome that can swell or shrink with the addition or removal of a denaturant resulting in protein unfolding or folding, respectively (*112*). Polymer-based capsules are more stable than liposomes and can encapsulate both hydrophilic and hydrophobic cargo, due to the amphiphilic nature of the copolymers. Membrane characteristics are easily manipulated by conjugating different functional groups to the polymer, beneficial for facilitating drug loading and drug delivery (*113*). Lastly, hybrid systems have been developed combining features of different types of expression platforms. A recent example was the development of eCells, where layer-by-layer polymer assembly was employed to encapsulate *E. coli*, followed by cell lysis resulting in encapsulation of a cell-free expression system with component concentrations similar to *in vivo* conditions (*114*).

1.6.3 HYDROGEL

Hydrogels provide an open system for the easy exchange of components while also increasing proximity to better mimic an *in vivo* environment. These hydrophilic, polymeric three-dimensional networks are composed of natural or synthetic molecules, such as RNA (*115*), DNA (*116*), agarose (*117*), clay (*118*), hyaluronan (*119*), or polyacrylamide (*120, 121*). In combination with a cell-free protein synthesis system, hydrogels have been employed in two ways: 1) as a template for gene expression, or 2) as a scaffold for protein binding, providing additional features of encapsulation.

The formation of a protein producing hydrogel, or p-gel, was first described by Park *et al.* (2009) (*116*). In this study, a gel micropad was formed by ligating X-shape and linear plasmid DNA

within a polydimethylsiloxane micromould (*116*). Upon incubation with a cell lysate from *E. coli*, wheat germ, or rabbit reticulocyte, protein synthesis was demonstrated for the expression of membrane proteins, kinases, and toxic proteins (*116*). In comparison to batch reactions, an increase in protein production of ~94-fold was reported. This improvement in protein synthesis was hypothesized to result from an increase in the rate of transcription due to the close proximity of genes in the hydrogel environment.

As a scaffold, hydrogels have also been functionalized with binding sites to immobilize specific proteins, solving issues of diffusion in bulk reaction conditions and increasing the proximity between components (*119, 121, 122*). For example, Zhou *et al.* (2018) localized his-tagged protein components of the PURE TX-TL system on a nickel nitriloacetic acid (Ni^{2+}NTA) functionalized polyacrylamide gel, resulting in long term protein expression of 11 days when supplied with a continuous flow of nutrients and substrates (*121*). Similarly, Lai *et al.* (2020) functionalized a hydrogel with anti-His-tag aptamers rather than nickel ions for improved biocompatibility, extending the lifetime of a TX-TL reaction to 16 days with a continuous flow of a nutrient-rich buffer and a microfluidic device (*122*). Heida *et al*. (2020) combined both functionalities of protein synthesis and immobilized components within a hyaluronan gel by localizing a DNA template for the expression of his-tagged protein, that could subsequently be immobilized on the gel in the presence of Ni^{2+} NTA moieties (*119*).

Building towards the creation of proto-organs, Bayoumi *et al.* (2017) investigated layered encapsulation within a hydrogel to mimic organelles within an extracellular matrix (*117*). Proto-organelles were formed through the immersion of aqueous droplets in an oil-lipid bath, which were then encapsulated in a hydrogel to form proto-cells. Low melt agarose was used as the hydrogel material due to its availability, low cost and biocompatibility. The incorporation of membrane proteins to form nanopores and the encapsulation of specific molecules resulted in programmed communication between different arrays of droplets within the hydrogel.

A relatively unexplored area of hydrogel research is the use of RNA building blocks. Huang *et al.* (2017) demonstrated the self-assembly of a RNA-hydrogel (*115*), which provides unique features of structural flexibility and direct protein synthesis from an mRNA template. However,

RNA-based hydrogels have not been further developed for application with cell-free systems. The investigation of different materials to build hydrogels have the potential to add novel features of programmable biological function, representing a future direction of hydrogel research.

1.6.4 LYOPHILIZATION

Lyophilization, or freeze drying, of cell-free reactions stabilizes the system for long term storage as opposed to traditional storage methods, which consists of flash freezing aqueous reactions and storing at -80°C (*123*). In the latter case, the activity of the system is lost over time, with a 50% reduction after storage for one week (*123*). Alternatively, cell-free reactions lyophilized on paper are stable up to one year and can be activated when needed (*124*). The lyophilized platform provides a valuable tool for biosensor development, on-demand protein production, and use in remote locations (*125*). The utility of lyophilization was demonstrated with the paper-based detection of Ebola (*126*) and Zika virus (*29*) which employed a lyophilized cell-free system to control an engineered genetic circuit. Specifically, Pardee *et al.* (2014 and 2016) used an RNA aptamer-based system, where a conformational change in the mRNA encoding a reporter protein was induced in the presence of a target RNA sequence, referred to as a toehold switch (*29, 126*). Overall, freeze drying has proven a useful tool for the deployment of cell-free reactions outside a laboratory or controlled setting in a safe manner.

1.7 RELEVANCE TO SYNTHETIC BIOLOGY

Cell-free systems continue to be instrumental in the study of biological processes and contribute to an increased understanding of how biological systems work. In conjunction with this, cell-free systems lead to foundational advances in synthetic biology, including for the synthesis of difficult-to-express proteins, prototyping of genetic parts and metabolic pathways, protein engineering, and the development of artificial cells.

1.7.1 DIFFICULT-TO-EXPRESS PROTEINS

In vivo recombinant protein expression is associated with issues of cytotoxicity, poor solubility, and instability for specific types of proteins, such as antibodies or membrane proteins. For example, induced expression of membrane proteins can lead to the undesirable formation of

inclusion bodies, protein aggregation, or the activation of signal transduction pathways within a cell (*127*). To overcome these obstacles, cell-free protein expression has been employed, providing a reduced and controllable environment for the synthesis of difficult-to-express proteins, enabling the analysis of complex protein structure and function (*127*). For example, cell-free protein expression can result in higher yields than is possible to achieve *in vivo*, providing the required starting material for structural analysis by crystallographic methods (*128*).

Membrane proteins, including G-protein coupled receptors (GPCRs), constitute over 60% of all known drug targets (*129*). GPCR's are highly relevant for the development of new drug therapies due to their accessibility at the cell surface and involvement in a diverse range of physiological processes, including a role in diseases such as diabetes and Alzheimers when mutated (*130*). The hydrophobic nature of membrane proteins requires the use of biomimetic support systems, such as liposomes or microsomes, or the addition of detergents, to ensure proper protein folding. Cell-free protein synthesis of functionally active GPCRs has been demonstrated for the human dopamine D2 receptor long isoform (*131*), the human histamine H1 receptor (*132*), and the β2 adrenergic receptor (*133*) using *E. coli* cell extract. Eukaryotic-based extracts contain the necessary machinery for post-translational modifications and for translocation into microsomes, providing a model system more closely related to humans. Cell extracts generated from insect (Sf21) cells have been employed to investigate ligand binding properties of the μ opioid receptor (*134*) and endothelin B-receptor (*135*). Thoring *et al.* (2017) demonstrated high yields of difficult-to-express proteins, including epidermal growth factor receptor (EGFR) and single chain antibody variable fragments (scFvs), using a CHO-based extract in a continuous exchange reaction environment (*136*). In the case of scFvs, this work reached high protein yields of 980 μg/mL, building towards larger scale industrial protein production. Cell extract-based systems can contain residual membrane components, due to incomplete removal of the cell membrane fraction from the cell lysate during processing, complicating functional studies for recombinantly produced proteins (*137*). Alternatively, the PURE system encapsulated within liposomes, provides a defined system with minimal contamination and has been used for the synthesis of a variety of membrane proteins including bacteriorhodopsin (*138*), F_OF_1-ATP synthase (*139*), and the SecYEG translocon (*140*).

Toxins represent another group of difficult-to-express proteins due to the negative impact on cell viability by inducing apoptosis when expressed *in vivo* or inhibiting gene expression, presenting a challenge for the study of uncharacterized toxins. Cell-free approaches provide a solution to this problem, allowing for the rapid synthesis and functional assessment of toxins, building towards the development of new diagnostic tools (*141, 142*). For example, Ramm *et al.* (2020) used a CHO cell lysate for the *in vitro* synthesis of a functionally active tripartite non-hemolytic enterotoxin (Nhe) from the opportunistic pathogen *Bacillus cereus* (*142*). This study examined pore-forming and cytotoxic activity of the compound to elucidate the toxins mechanism of action, which is currently unknown (*142*). Overall, these studies demonstrate the wide-range of proteins, with primarily therapeutic relevance, that can be expressed using cell-free approaches.

1.7.2 PROTOTYPING

The engineering of cellular systems to perform a specific function in a reliable and predictable manner requires extensive optimization and lengthy DBTL cycles. Gene expression levels need to be fine-tuned and are dependent on the genetic elements used, including plasmid copy number, promoter, RBS, and transcriptional terminator. Therefore, it is important to know the activity and function of each genetic element, and the influence varying contexts have on system performance to facilitate the rational design and construction of complex systems that behave as expected *in vivo*.

Cell-free systems provide a valuable prototyping tool for the characterization of genetic parts (*143*), genetic circuits (*144-146*), and metabolic pathways (*147, 148*), before executing *in vivo,* ultimately accelerating the DBTL cycle. In this manner, time-consuming cell culturing, transformation, and genetic cloning steps are bypassed. Previous studies have demonstrated good agreement between *in vitro* and *in vivo* characterization of DNA and RNA regulatory elements (*149, 150*), CRISPR elements (*151*), and synthetic oscillators (*151*). Linear DNA, including synthesized DNA or PCR products, can be used as direct inputs, further quickening the speed of characterization (*152*). Lastly, cell-free systems are amenable for high-throughput analysis, as demonstrated by genetic circuit optimization utilizing microfluidics (*153*).

When accompanied by computational modeling, the design and predictability of biological systems is improved, making prototyping easier to do (*154*). *In silico* modeling of TX-TL processes has helped identify competition between resources (*155*) and the accumulation of inhibitory metabolites (*156*), guiding optimization of the reaction environment. Based on the PURE system, Matsuura *et al.* (2017) modeled the synthesis of a small peptide to study the dynamic features of protein synthesis (*157*). From their model, potential bottlenecks were identified, including a slow dissociation of the 70S ribosome into the 30S and 50S subunits, impacting ribosome turnover and limiting the rate of protein synthesis. Overall, computational modeling coupled with prototyping enables rapid design and testing of biological components, as well as to provide a better understanding of the kinetic and thermodynamic parameters (*158*).

1.7.3 PROTEIN AND RIBOSOME ENGINEERING

Directed evolution of proteins aims to accelerate and direct the process of natural selection towards a user-defined purpose, including for improved catalytic activity or binding affinity. A gene encoding a POI is subject to mutagenesis for the creation of a library of variants, followed by variant selection through activity assays, and candidate amplification to generate a template for the next round of mutagenesis and selection. Cell-free approaches to directed evolution allow for the entire sequence space to be explored in the absence of cellular constraints or inherent bias, and expands the choice of reaction conditions tested, including the buffer composition and reaction temperature.

Early reports in the late 1990s described *in vitro* selection and evolution of proteins through methods referred to as ribosome display (*159*) and mRNA display (*160*). In ribosome display, genotype (DNA/RNA) is connected to phenotype (protein) through a linker region encoded at the end of the sequence. Following translation, the linker remains attached to the peptidyl tRNA, allowing for the POI to protrude from the ribosome and correctly fold. This results in the formation of a ribosome-POI-mRNA complex, which is selected for upon binding to a surface-immobilized target, allowing for the mRNA to be recovered for another round of selection. mRNA display follows a similar principle, but rather the POI is linked to the mRNA via a puromycin linkage. Ribosome and mRNA display has been applied for the directed evolution of proteins with therapeutic relevance

including antibody fragments (*161, 162*) and the identification of small peptides that bind to hepatis C virus envelope 2 protein (*163*).

A significant engineering challenge is the directed evolution of ribosomes, for unlocking novel functions and expanding the repertoire of amino acid building blocks. To overcome viability limitations *in vivo*, *in vitro* methods have provided a valuable approach, including reconstitution of the 30S and 50S ribosomal subunits (*164, 165*). Recently, Hammerling *et al.* (2020) combined techniques of "*in vitro* integrated synthesis, assembly, and translation (iSAT) with ribosome display" to synthesize mutant ribosomes resistant to the antibiotic clindamycin (*166*). Modifying the ribosome in a controlled *in vitro* environment helps to elucidate key aspects of translation. Other aspects of engineering the translation machinery include incorporating non-canonical or unnatural amino acids (*167*) and the creation of a six-alphabet code which is more tolerable in a cell-free environment (*168*). Overall, the open nature of a cell-free system provides an optimal environment for engineering novel biological functions.

1.7.4 ARTIFICIAL LIFE

Biological processes are highly complex, involving a network of interconnected pathways. It is critical to understand how these pathways relate to one another and ultimately, how a living system works, to engineer biology for a user-defined purpose. Two complementary approaches to synthetic biology have been applied for the study of biological systems, referred to as top-down or bottom-up (*169*). A top-down approach aims to reduce the complexity of biological systems through methods such as genome editing, simplifying a living system towards the creation of a minimal cell (*170*). Alternatively, the objective of bottom-up synbio is to build a living system from scratch, reconstituting biological processes from individual components to create an artificial cell (*171*). The latter approach follows the idea that a greater understanding of how a cell functions is only achieved if a cell can be built by scratch (from the Richard P. Feynman quote "What I cannot build. I do not understand"). Cell-free systems support this approach, providing the tools to reconstruct key aspects of what constitutes life. Creating a synthetic cell that completely mimics the dynamics of life is a challenging task, however immense progress has been made over the years, including

advances in compartmentalization, genetic circuit design, self-replication, energy regeneration, metabolism, communication, sensing, and motility (*91, 172, 173*).

Natural cell elements of compartmentalization and gene expression have been recapitulated and extensively demonstrated through liposome encapsulation of TX-TL machinery for the synthesis of a POI or of purified enzymes to execute a metabolic pathway (refer to *Section 1.6.2*). More complex genetic circuitry has been encoded for the communication between genetic modules, mirroring the coordinated interaction of living cells (observed in both multicellular and unicellular organisms) (*174*). Adamala *et al.* (2017) demonstrated programmed communication between different populations of "synthetic cells (syncells)", where two parts of a genetic circuit were compartmentalized into liposomes creating distinct modules; a sensor and a reporter module (*109*). Other groups have also engineered coordinated biological function by separating genetic circuits into "sender and receiver cells", following similar design principles (*94*) .

The ability to sustain cellular processes is a critical component of what constitutes life. Artificial cells require energy regeneration mechanisms to provide the necessary power to maintain cell-like function. ATP is considered the main energy currency of a cell. Therefore, ATP regeneration has been a key focus to power an artificial cell by exploring ways to convert light or chemical energy into ATP. Berhanu *et al.* (2019) created a photosynthetic artificial cell that converted light energy into ATP, by integrating bacteriorhodopsin and F-type ATP synthase (F_oF_1) into a liposome membrane (*138*). This study showed successful ATP generation, that was also used as a substrate in transcription and to drive protein synthesis (*138*). In bulk conditions, Opgenorth *et al.* (2017) developed an ATP rheostat to control ATP levels by monitoring the free phosphate concentration (*175*). Two complementary enzymatic pathways were created that both convert glyceraldehyde-3-phosphate into 3-phosphoglycerate, where one pathway generates ATP controlled by the presence of free phosphate, and the other does not produce an additional ATP.

Overall, breakthroughs are being made that demonstrate life-like functionality in a synthetic system built from scratch, as demonstrated by the select examples discussed here. By building an artificial cell and reconstituting aspects of a living system, a greater understanding of the fundamental physical and chemical processes is gained, as well as insights into the origins of life.

This knowledge can further be applied to the development of biotechnology inspired by nature, as well as open the door to novel systems and processes not observed in nature.

1.8 BIOTECHNOLOGY APPLICATIONS OF CELL-FREE SYSTEMS

Cell-free systems have proven a valuable resource for the development of biotechnology that has a real benefit for society, including sustainable and transformative advancements in industrial manufacturing, environmental biosensing and bioremediation, medical biotechnology, and education.

1.8.1 INDUSTRIAL BIOMANUFACTURING

Bioengineering approaches to industrial manufacturing have the potential to overcome limitations of classical chemical based approaches by providing a biocompatible, inexpensive, and environmentally-friendly alternative. Previous work has exemplified this potential by engineering bacteria to produce commodity chemicals and high value products, including biofuel (*176*), bioplastic (*177, 178*), and cement (*179*). Although these approaches have demonstrated the promise of biotechnology, integration with current industrial infrastructure has been difficult stemming from concerns associated with the use of GMOs and the corresponding environmental impact, as well as sensitivity to working conditions, such as high temperature and pressure, and the types of solvents used (*180*). As an alternative, cell-free systems are inherently non-living (i.e. non-GMO), and allow for rapid DBTL cycles, flexible engineering, incorporation of toxic components, and improved control (*35, 46, 181*). For example, properties of a cell-free system can be rationally designed and finely tuned, including the activity and thermal stability, making cell-free methods an appropriate solution for integration with existing industrial processes.

A common approach to cell-free biomanufacturing is the reconstruction of metabolic pathways using recombinantly purified enzymes. Opgenorth *et al.* (2016) designed an artificial reaction pathway for the conversion of glucose to polyhydroxybutyrate, demonstrating the feasibility of a cell-free expression system for the cost-effective production of bioplastic (*178*). Building on this, the same research group constructed a biosynthetic pathway comprising of 27 purified enzymes for the production of monoterpenes, a class of industrially relevant metabolites used in

the production of food, cosmetics, pharmaceuticals, and biofuels (*182*). The reaction pathway was designed to be highly amenable for the synthesis of different types of monoterpenes facilitated by the exchange of a terpene synthase at the final step of the process for the production of a specific compound, such as limonene, pinene, or sabinene (*182*).

In addition to purified reaction components, crude cell extracts have also been used for industrial biomanufacturing. Through the combination of *E. coli* cell lysates enriched with specific enzymes, Dudley *et al.* (2015) built a 20 component enzymatic biosynthesis pathway for the production of limonene from glucose (*183*). Using a similar method, Cassini *et al.* (2018) demonstrated the value of cell-free protein synthesis for the rapid production of industrially relevant, but also toxic, compounds as a part of a pressure test for evaluating the capabilities of bioengineering methods and the ability to quickly respond in times of need (*184*). Furthermore, Yi *et al.* (2018) created a hybrid system, consisting of cell lysates from *E. coli* combined with cyanobacteria for the synthesis of (R,R)-2,3-butanediol (2,3-BD) from starch demonstrating a novel approach to industrial biomanufacturing (*185*).

1.8.2 ENVIRONMENTAL BIOTECHNOLOGY

Cell-based biotechnology has been developed for environmental biosensing and bioremediation, but there are concerns associated with environmental release and control (*186*). As an alternative, cell-free systems provide a safer solution, since currently these systems are not capable of replication or evolution. Cell-free expression can be executed in an open system allowing for the continuous exchange of components, which is a desirable feature for environmental biosensing, or contained within a capsule to sequester contaminants for removal and subsequent degradation as a bioremediation tool. Depending on how the system is engineered, cell-free conditions are potentially more tolerable to a broader range of environmental conditions and the reaction environment is tunable for handling varying temperatures and solvents. Growing environmental concerns associated with xenobiotic compounds can also be addressed due to the engineerability of a cell-free system for detecting a wide range of compounds.

Cell-free biosensors for environmental purposes have primarily been developed for the detection of contaminants in water. Thavarajah *et al.* (2020) engineered a transcriptional biosensor

for the detection of fluoride in unprocessed groundwater, demonstrating a field deployable biosensor sensitive at detecting health relevant levels (*187*). The biosensor, executed in an *E. coli* cell extract, consisted of a *Bacillus cereus* crcB fluoride riboswitch controlling the expression of a colorimetric enzyme, catechol (2,3)-dioxygenase (C23DO). C23DO oxides the colorless substrate, catechol, to produce a yellow compound, 2-hydroxymuconate semialdehyde, which is easily visible by the naked eye providing a simple approach for fluoride identification. The detection of atrazine, a common surface water pesticide, was described by Silverman *et al.* (2020) (*188*). In this work, the authors employed a previously described cell-free biosensor for the detection of cyanuric acid, which was initially applied in unfiltered pool water (*189*), with a metabolic pathway to convert atrazine to cyanuric acid. To minimize the cellular burden of expressing all required enzymes in a single strain, multiple extracts were combined where each enzyme required for the biosynthesis of cyanuric acid from atrazine was expressed in a different cell strain. This provided a modular approach to biosensor construction. In general, basic design principles for biosensor design are followed, providing a level of modularity, which can be applied to the detection of a wide range of compounds using cell-free systems.

The next step following detection of contaminants in water, is the removal of these toxic and poisonous compounds. With respect to the degradation of cyanide, Nallapan Maniyam *et al.* (2015) prepared a cell extract from Gram-positive Rhodococcus UKMP-5M and demonstrated 80% degradation of 20mM potassium cyanide in 80 minutes (*190*). Overall, Rhodococcus UKMP-5M was effective at cyanide removal but would require additional development for large scale water remediation due to the initial tests being conducted in a closed, sealed environment which is not representative of field use. Another target for bioremediation is the removal of heavy metals in water, such as hexavalent chromium (Cr(VI)), a toxic and carcinogenic compound (*191*). To sequester and remove Cr(VI), Panda *et al.* (2014) developed calcium alginate beads modified with a cell-free extract form *Enterobacter aerogenes T* that is resistant to Cr(VI) (*191*). This organism contains a chromate reductase enzyme that converts Cr(VI) to Cr(III), resulting in a change in the redox environment, which can be electrically monitored providing a mode for biosensing, as well as bioremediation tool due to conversion to a less toxic compound (*191*). Lastly, the biosynthesis

of enzymes for water treatment is another application of cell-free environmental biotechnology. Li *et al.* (2016) synthesized a multicopper oxidase to treat wastewater decolorization in an *E. coli* cell lysate, overcoming issues of low yields and poor solubility when synthesized *in vivo* (*192*).

1.8.3 MEDICAL BIOTECHNOLOGY

The application of cell-free biomanufacturing for the production of medically relevant compounds has been explored due to the many advantages discussed in *Section 1.8.1*. When applied to the production of pharmaceuticals, cell-free protein synthesis of established therapeutics, can improve yield and simplify the production process compared to *in vivo* methods, and also provides a useful tool for the synthesis of novel therapeutics. Antibody production is a common application of cell-free protein synthesis methods. For example, CHO cell lysates have been optimized for the synthesis of monoclonal antibodies in high yield (*136*), and recombinant streptokinase for the treatment of blood clots (*193*), while insect cell lysates have been successful at producing scFvs (*194, 195*). Other applications include for the synthesis of colicins, an antimicrobial protein as an alternative to conventional antibiotics (*196*), the cytotoxin protein onconase as a cancer therapeutic (*197*), and the therapeutically relevant cannabinoid precursors, cannabigerolic acid and cannibigeroviarinic acid (*198*). When combined with on-chip synthesis and microfluidic technology, cell-free systems for point-of-care biomanufacturing is possible (*199*).

Cell-free biosensors have also been developed for clinical applications. Most notably, Pardee and colleagues developed rapid, portable paper-based biosensors for the detection of Ebola (*126*) and Zika virus (*29*). Voyvodic *et al.* (2019) created a modular workflow for creating a wide range of biosensors capable of detecting different chemical compounds including hippuric acid in urine (*200*). More recently, paper-based nucleic acid biosensors have been developed for the detection of SARS-CoV-2 RNA in saliva (*201*). Overall, cell-free biosensors are expanding to detect a variety of analytes with therapeutic relevance.

1.8.4 EDUCATION

Hands-on, practical demonstrations are often not a feasible approach for teaching the basic principles of biology to K-12 students due to the specialized equipment and expertise required, not to mention the challenges associated with handling GMOs in a classroom. A solution to this

problem has been developed using lyophilized, cell-free reactions creating synbio educational kits that are simply activated with the addition of water and input DNA, called BioBits™. Through sensory engagement, fundamental concepts of protein expression, enzyme catalysis, and biomaterial properties are taught with the BioBits™ Explorer kit (*202*). These concepts are demonstrated by the production of fluorescent proteins, enzyme generated smells, and enzyme cross-linked hydrogels (*202*). Low-cost equipment and supporting curriculum has been developed, detailed in BioBits™ Bright (*203*). Cell-free expression systems employed in the BioBits™ kits provide a modular, easy to use and affordable approach for introducing synbio to the next-generation of bioengineers and researchers.

1.9 OUTLOOK AND THESIS OBJECTIVES

Cell-free synbio opens the door to new opportunities for biotechnology, in addition to furthering our understanding of how biological systems work. To realize this potential and see the benefit to society, it is important to continue building cell-free technology and address current limitations that may block progress and real-world application. Highly purified *in vitro* reconstituted cell-free systems provide unique advantages over engineering whole cells, as well as offer different design features opposed to cell lysates. However, *in vitro* reconstituted TX-TL systems are also associated with challenges that impede those new to the field and restrict how they are used.

In February of 2020 the National Institute of Standards and Technology (NIST) released a report summarizing the findings of a workshop focused on addressing the challenges of cell-free synbio (*204*). Their report recommended the development of new methods to tackle issues of variability and reproducibility, as well as the need to develop common protocols (*204*). Along these lines, we identified the following questions to be addressed: *how can we improve access and lower the barrier to cell-free synbio? What tools are available to characterize the composition of cell-free protein synthesis systems? How can we improve cell-free systems to reach comparable, or better, protein yields achieved in vivo?* Lastly, increased accessibility is accompanied by societal implications that may not always be positive. *How do we ensure safe and responsible use of next-*

generation biotechnology as it rapidly develops? Therefore, four main research goals were developed:

1) Produce an *in vitro* reconstituted cell-free expression system in-house and provide detailed guidelines for system preparation, including troubleshooting strategies.

2) Examine the ribosome and TX-TL protein composition to understand system limitations and sources of variability, providing a tool to guide system optimization.

3) Demonstrate improved protein synthesis yields by targeting ribosome-associated processes.

4) Assess the policy implications of cell-free systems and next-generation synbio technologies.

Overall, this thesis aims to improve the ease and simplicity at which purified *in vitro* reconstituted TX-TL systems are obtained, understand the challenges associated with in-house preparation, characterize the composition of the system to guide optimization, and develop ways to improve system functionality. Lastly, to complement the experimental aspects of my work, it was my goal to assess the emerging regulatory challenges associated with next-generation synbio and cell-free biotechnology. Altogether, the work reported here contributes to the ongoing efforts towards improving the performance and accessibility of cell-free protein synthesis.

CHAPTER 2 - DEVELOPMENT OF A HIGHLY PURIFIED *IN VITRO* RECONSTITUTED CELL-FREE EXPRESSION SYSTEM

2.1 PREFACE

Chapter 2 has been written in manuscript form for submission to an open-access methods-based journal detailing the preparation and validation of a highly purified *in vitro* reconstituted TX-TL system for accessible cell-free protein synthesis. *In vitro* reconstituted TX-TL systems are challenging to produce in-house and, in the case of sub-optimal gene expression, are difficult to troubleshoot. To address these challenges, we provide guidelines for producing a cost-effective, modular, and functional cell-free protein synthesis system. The manuscript describes in detail methods for 1) preparing an energy solution, 2) individual protein expression, purification, and characterization of TX-TL factors, and 3) large-scale ribosome purification. Furthermore, the utility of a validation tool to benchmark gene expression on the transcriptional and translational level was demonstrated. This work provides valuable information for lowering the barrier of cell-free systems to those new to the field, facilitating the broader use of cell-free protein synthesis.

Contributions of Authors: The project was designed by Dr. Hans-Joachim Wieden and myself. TX-TL protein expression, purification, and characterization was done by myself. Mass spectrometry was performed by the University of Lethbridge proteomics facility (Fan Mo), while I conducted subsequent data analysis. The complete *in vitro* reconstituted TX-TL system was prepared and tested by myself. I wrote the manuscript.

2.2 INTRODUCTION

In vitro TX-TL systems produce RNA and protein outside of a living organism, providing an alternative approach to recombinant protein synthesis in cells. Also referred to as cell-free, these systems utilize cell lysates or reconstituted translation machinery from individually purified components, providing an engineerable reaction environment (*45*). Both types of cell-free systems offer several advantages compared to cell-based methods, including a reduced bio-contamination

risk, the ability to incorporate unnatural amino acids, and the tolerance of toxic compounds (*35, 46*). In a cell-free system, all of the energy and resources are directed towards its designed function and the system does not compete with natural cellular metabolism, as is the case with living systems (*35*). This feature is even more evident for purified reconstituted systems, which have a well-defined composition consisting of the minimal components required for gene expression, reducing the potential for cross-talk or unanticipated reactions. Overall, cell-free systems are highly amenable for applications in biotechnology, as well as a useful tool for studying the fundamental processes of gene expression (*181*).

The first highly purified *in vitro* reconstituted cell-free expression system was reported in 2001 by Shimizu *et al.*, referred to as the protein synthesis using recombinant elements (PURE) system (*68*). The described PURE system consisted of 31 TX-TL factors individually purified by nickel affinity chromatography, including aminoacyl-tRNA synthetases (aaRS's), initiation factors (IFs), elongation factors (EFs), release factors (RFs), ribosome recycling factor (RRF), methionyl tRNA transformylase (MTF), inorganic pyrophosphatase (PPiase), and T7 RNA polymerase (RNAP), as well as ribosomes isolated by sucrose-density-gradient centrifugation. Additional components required for energy regeneration, specifically creatine kinase (CK), myokinase (MK), and nucleotide diphosphate kinase (NDK) were included. The required substrates, such as tRNAs, NTPs, creatine phosphate, amino acids and other cofactors, were also added.

The PURE system is now commercially available from New England Biolabs (NEB) as the PURExpress® kit. It consists of a Solution A and B that produce a protein-of-interest (POI) when combined with template DNA. Other commercial *in vitro* protein synthesis systems based on the PURE system are also available, including PURExpress® from GeneFrontier Corporation, where the protein component does not contain affinity tags, and the Magic™ PURE System from Creative Biolabs that has been optimized for membrane protein expression. Although commercial systems provide a simplified approach to *in vitro* protein synthesis, they are expensive to purchase, and the modularity of the system is lost. An exception to this is three specialized PURExpress® kits that separate out the RFs, tRNA and amino acids, or ribosomes (available as PURExpress® ΔRF123, PURExpress® Δaa/tRNA, PURExpress® ΔRibosomes). Alternatively, the PURE system can be

prepared in-house but is laborious and challenging to produce. In both cases, these systems also result in a low protein yield in comparison to cell extracts and *in vivo* methods (*205*), presenting additional barriers to their widespread use.

To address limitations of the commercial systems, research has expanded on easy and cost-effective approaches to produce the PURE system in-house. Direct insertion of his-tag sequences into the genetic loci of *E. coli* enabled the co-purification of the translation machinery from 6 cell strains (including an additional elongation factor; EF-4), resulting in the ePURE system (*69*). ePURE required fewer purification steps, but demonstrated approximately a tenth of the commercial protein synthesis activity (*69*). Shepherd *et al.* (2017) simplified the purification process by constructing three expression plasmids encoding 30 of the translation factors (not including EF-Tu, T7 RNAP, and the energy regeneration components) (*70*). Referred to as PURE 3.0, this approach required three protein expression and purification steps. When combined with the other translation machinery components, similar yields of the protein DHFR were produced compared to the PURExpress® system. However, when taking this approach one step further by constructing a 30-cistron plasmid encoding the TX-TL factors, the resulting system was not capable of gene expression due to low protein yield generated from the resulting expression vector.

In the same year, the TraMOS method was described by Villarreal *et al.* (2017) (*71*). In this work, a single cell culture, lysis, and purification method was demonstrated using engineered microbial consortia, where multiple *E. coli* strains were engineered to express between one and three of the 32 translation factors (including EF-4, but not including T7 RNAP or energy regeneration factors). Similarly, Lavickova *et al.* (2019) developed a OnePot purification method where *E. coli* cell expressing each factor were co-cultured, followed by a single lysis and purification step (*72*). This procedure included the purification of 36 proteins (TX-TL factors, and energy regeneration components) and described a simple method to purify ribosomes. Both systems were capable of protein synthesis; the OnePot method produced similar protein levels compared to the PURE system, while a fourth of the protein levels was produced using the TraMOS approach.

ePURE, PURE 3.0, TraMOS, and OnePot purification methods highlight the progress towards accessible cell-free protein synthesis by offering streamlined approaches to produce *in vitro*

reconstituted TX-TL systems in-house. Due to differences in how the systems are prepared, each approach has their own limitations. For example, the composition of ePURE, PURE 3.0 and TraMOS is less modular, since multiple proteins are expressed in a single plasmid or cell strain. Control over the concentration of each component is also challenged by purifying multiple proteins in a single step, such as for the OnePot method. Furthermore, questions arise regarding reproducibility of preparing the system given the inherent variability of different amounts of protein binding to the nickel column during a single purification step. Therefore, the application or end-use of the system and desired design features informs which method should be used (i.e. there is a trade-off between individually purifying each component and a single purification step). In addition to TX-TL proteins, efficient and cost-effective methods are also required to prepare the energy solution and ribosomes.

Challenges remain regarding the production of a highly functional, modular, and cost-effective *in vitro* reconstituted cell-free expression system. Due to the number of components involved it is difficult to prepare the system in-house and troubleshoot the system if any issues arise, especially for those new to the cell-free field. Furthermore, detailed methods for system preparation is lacking. In the interest of working with a flexible and modular expression system, it was our goal to individually purify each component, understand common areas of difficulty, and improve the preparation process. Here we summarize the purification and characterization of each TX-TL protein, the preparation of a working energy solution, and large-scale purification of ribosomes. A fluorescent-based diagnostic tool consisting of Spinach RNA-tagged enhanced yellow fluorescent protein (EYFP) was designed and tested to characterize transcription (Spinach RNA) and translation (EYFP). We provide a detailed cost analysis and compare our in-house TX-TL system to the commercial PURExpress®, as well as to other reported systems, and discuss the benefits of producing the system in-house. Lastly, we propose approaches to improve cost-effectiveness and functionality of the cell-free expression system. Overall, this work builds towards decreasing the barrier to use cell-free expression systems, enabling applications in biotechnology development.

2.3 METHODS

2.3.1 REAGENTS, PLASMIDS, AND STRAINS

Details of the chemicals and reagents used in this study are summarized in Tables A2-1 to A2-5, or were obtained from BioBasic, unless otherwise specified. Plasmids encoding TX-TL proteins were synthesized by Genewiz, Integrated DNA Technologies (IDT), or BioBasic and sub-cloned into pSB1C3, pUC57, or pJET. The plasmid encoding T7 RNAP was provided as a gift-in-kind from Dr. Ute Kothe (University of Lethbridge, Canada). EF-Tu and EF-G were previously sub-cloned into pET21a and pET28a, respectively. The original plasmids encoding EF-Tu and EF-G were provided by Dr. Barend Kraal (Leiden University, Netherlands) and Dr. Marina Rodnina (University of Göttingen, Germany), respectively. Gene sequence, originating organism, plasmid, antibiotic resistance, and expression strain for each protein is summarized in Table A2-6. The validation construct (Spinach-tagged EYFP) was synthesized as a gBlock gene fragment from IDT and sub-cloned into pJET (CloneJET PCR Cloning Kit, Thermo Fisher Scientific) (Table A2-7). All constructs were sequence confirmed by Genewiz. Plasmid DNA was amplified in *E. coli* DH5α and purified using the EZ-10 Spin Column Plasmid DNA Miniprep Kit. Purified and sequence confirmed plasmid DNA was transformed in *E. coli* BL21-Gold (DE3) for protein expression.

2.3.2 EXPRESSION AND PURIFICATION OF PROTEINS

A single standard purification protocol was followed for all 36 TX-TL proteins, including the alpha and beta subunit for two of the aaRS's, glycine tRNA synthetase (GlyRS) and phenylalanine tRNA synthetase (PheRS), resulting in 38 purifications total. TX-TL proteins were hexahis-tagged at the N- or C-terminus, depending on previous literature (*69*). *E. coli* cells containing TX-TL plasmid DNA were grown in Luria Broth (LB) media supplemented with 35 µg/mL chloramphenicol, 100 µg/mL ampicillin, or 50 µg/mL kanamycin depending on the encoded antibiotic resistance. TX-TL protein expression was induced at an OD_{600nm} of approximately 0.6 with isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. 3-hours after induction, cells were harvested by centrifugation at 5 000 xg for 20 min, flash frozen with liquid nitrogen, and stored at -80°C. To purify the protein, the cell pellet was thawed on ice and resuspended in 5 mL/g (cells) of Buffer A (Table A2-2). To lyse the cells, 1 mg/mL of lysozyme was slowly added at 4°C and

incubated for 30 min, followed by the addition of 12.5 mg/g (cells) of sodium deoxycholate at 4°C and incubated for 60 min. The cell lysate was then centrifuged at 30 000 xg for 30 min at 4°C. The TX-TL protein-containing supernatant was applied to 3 mL of Ni^{2+} Sepharose resin (GE Healthcare), which was previously equilibrated with Buffer A, and incubated for 1-hour at 4°C. To remove the unbound fraction, centrifugation at 500 xg for 2 min was performed. To remove any non-specifically bound protein, the resin was incubated with 40 mL of Buffer B (Table A2-2), followed by centrifugation at 500 xg for 2 min and decanting of the supernatant. This wash step was repeated an additional 3 times. To elute the protein, 4 mL of Buffer E (Table A2-2) was added to the resin, incubated at 4°C for 5 min and centrifuged at 500 xg for 2 min. This was repeated for a total of 10 elution's, which were subsequently pooled, and concentrated using a spin column (Vivaspin 10/30/50, GE Healthcare). To remove any contaminating protein and increase the purity of the protein of interest, the concentrated sample was loaded with a flow rate of 1.0 mL/min onto a Superdex 75 XK26/100 (GE Healthcare, S6657 and GE29321909) or Superdex 200 GL (GE Healthcare, 17517501) size exclusion column equilibrated with $TAKM_7$ buffer (Table A2-2). Column choice was dependent on the size of the protein of interest. Peak fractions were collected, pooled and concentrated using ultra filtration (Vivaspin 10/30/50, GE Healthcare). The purified protein was aliquoted, flash frozen in liquid nitrogen, and stored at -80°C. The protein concentration was determined by using the molar extinction coefficients (from the ProtParam tool using the amino acid sequence) of the respective protein, summarized in Table A2-8, and spectroscopic measurements at 280 nm (BioDrop DUO UV/VIS Spectrophotometer, Biochrom, Ltd.). For proteins without tryptophan residues (indicated in Table A2-8), the concentration was determined by densitometry analysis using ImageJ software to quantify sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) band intensity relative to a protein standard of known concentration (*206*). Protein purity was confirmed by 10, 12, or 15% SDS-PAGE, mass spectrometry analysis (detailed in section 2.3.4), and A260 nm/A280 nm spectroscopic analysis (Table A2-8). A summary of the individual protein expression and purification is provided in Table A2-9.

2.3.3 MASS SPECTROMETRY SAMPLE PREPARATION AND DATA ANALYSIS

Protein samples were prepared following an in-gel digestion method to denature proteins. Protein samples were resolved on a 12% SDS-PAGE, stained with Coomassie Blue R-250 for protein visualization, and excised from the gel. SDS-PAGE gel samples were first washed with H_2O and dehydrated with acetonitrile (ACN). Samples were then reduced with 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 at 56°C for 50 minutes and alkylated with 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 20 minutes in a thermoshaker at 26°C, 1050rpm. After two washes with 100 mM NH_4HCO_3, the samples were digested with 12.5 ng/ml trypsin solution with 41.7 mM NH_4HCO_3 and 4.17 mM $CaCl_2$ at 37°C overnight. Digested peptides were extracted with ACN, and dried. Then peptides were reconstituted in 5% formic acid (FA).

Data was acquired on a Thermo Scientific Orbitrap Fusion mass spectrometer coupled with a Thermo Scientific EASY-nLC 1000. 450 ng of reconstituted peptide samples were loaded on a 0.075 mm x 500 mm Accucore 150 C18 with 2.6 µm particles at a flow rate of 200 nl/min with a 20 mm Acclaim PepMap 100 C18 trap column. Samples were separated on a short gradient from 3% to 13% B (7 min), 13% to 44% B (50 min), 44% to 100% B (5 min) and 100% B (11 min), or long gradient from 3% to 13% B (15 min), 13% to 44% B (100 min), 44% to 100% B (10 min) and 100% B (10 min). Solvents contained A: 0.1% FA in water, and B: 0.1% FA in 80% ACN. MS/MS methods were programmed in the data dependent acquisition top speed mode, MS1 surveys were acquired using orbitrap and MS2 surveys were acquired using ion trap. For MS1 scans, the scan range was from 300 to 1500 m/z, with resolution at 120,000, and automatic gain control (AGC) target at $2x10^5$, 12 s exclusion duration. Ions with charge states 2+ to 7+ were subjected to MS/MS acquisition with high-energy C-trap dissociation (HCD). For MS2 HCD scan at 1.2 s cycle time, the isolation window was 1.2 m/z, the collision energy was 30%, the AGC target was $1x10^4$, and the maximum injection time was set to 35 ms.

Raw data were loaded into Maxquant 1.6.1.0, using default settings with iBAQ and match between run enabled. To identify peptides, the reference data set UP000000625 *E. coli* strain K12 was used. The database was supplemented with protein sequences for CK, PPiase, and T7 RNAP,

as the coding sequences are derived from organisms other than *E. coli* (summarized in Table A2-6).

Data was filtered as described in Smits *et al.* (2013) (*207*). In brief, contaminants, reverse hits, and proteins with unique peptide counts less than 1 and/or with counts less than 2 were removed. Protein relative abundance was determined using the iBAQ method. The iBAQ value represents the sum of all peptide intensities for each protein divided by the number of theoretical peptides produced from a trypsin digestion (*208*). When normalized by the sum of all iBAQ values, protein relative abundance is inferred, providing a measure of protein purity (Table A2-8). Contaminating proteins that account for ≥1% of the sample are summarized in Table A2-10.

2.3.4 PURIFICATION OF RIBOSOMES

Ribosomes were purified from *E. coli* MRE 600 following methods described in Rodnina *et al.* (*209*) Composition of the buffers used for the purification are summarized in Table A2-3 and A2-4.

2.3.5 PREPARATION OF ENERGY SOLUTION

The composition of the in-house energy solution is defined in Table A2-1. Methods by Shimizu and Ueda (2010) were followed, with slight modification (*210*). In brief, a concentrated stock of each component was prepared, facilitating the preparation of a 4X concentrated energy solution (Table A2-1). The energy solution was prepared in a tris, ammonium chloride, and potassium-based (TAK-based) buffer. Amino acids were prepared following methods described by Caschera and Noireaux (2015) (*211*). Two approaches were tested to prepare the folinic acid: 1) in TAK buffer, based on buffer preference, and 2) in HCl, as described in Shimizu and Ueda (2010) (*210*). All components, except tRNA and amino acids, were filter sterilized. Two approaches to prepare NTPs were also tested: 1) as described in Shimizu and Ueda (2010) (*210*), and 2) following an in-house established protocol for NTPs used in an *in vitro* transcription (IVT) reaction. Although the pH was adjusted for individual components, such as NTPs to 7.0, it was important to set the pH of the final assembled solution to 7.0.

2.3.6 *IN VITRO* TRANSCRIPTION REACTION

IVT reactions were prepared with 50 ng/µl of template DNA, 40 mM Tris-HCl pH 7.5, 15 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 2.5 mM of each NTP (ATP, cytosine triphosphate (CTP), uridine triphosphate (UTP), and GTP), 5 mM GMP, 0.01 U/µL inorganic pyrophosphatase, and 0.5 U/µL of the RNase inhibitor RiboLock (Thermo Fisher, E00381). Recombinantly purified T7 RNAP, either from this study or provided as a gift-in-kind from the research group of Dr. Trushar Patel (as a positive control), was added to a final concentration of 0.77 µM. The reaction was incubated at 37°C for 16-hours, followed by DNase I digestion of the template DNA incubated at 37°C for an additional 1-hour. Samples were removed before and after the overnight incubation and added to 3 µL of 3M NaOAc pH 5.2 to stop the reaction and following DNase I digestion. The samples were analyzed on a 12% 8 M urea PAGE gel to confirm RNA production.

2.3.7 IN-HOUSE TX-TL SYSTEM AND PUREXPRESS® REACTIONS

The PURExpress® kit from New England Biolabs (E6800) provided a positive control to compare to the in-house TX-TL system. The user manual was followed with slight modifications. In brief, 10 µL of Solution A, 7.5 µL of Solution B, 250 ng of plasmid DNA, and 0.5 µL of RNase Inhibitor (RiboLock, Thermo Fisher, E00381) was combined with milliQ H_2O for a final reaction volume of 25 µL. Reactions were incubated overnight at 37°C and placed on ice after 16-hours to stop the reaction. The 16-hour incubation period is longer than the recommended 2-4 hours but was required to ensure complete maturation of the fluorescent reporter protein. In each set of experiments, a negative control was included, where milliQ H_2O was added in replacement of the template DNA. Reactions were performed in triplicate, unless otherwise indicated.

Similarly, the in-house TX-TL components were combined and incubated at 37°C overnight for 16-hours. The composition consisted of 6.25 µL of the Energy Solution, approximately 7 µL of the Factor Mix (purified protein was combined to obtain a final concentration for each factor as described in Lavickova *et al.* (2019)), 60 pmoles of ribosomes, 250 ng of plasmid DNA, and 0.5 µL of RNase Inhibitor (RiboLock, Thermo Fisher, E00381) with milliQ H_2O for a final reaction volume of 25 µL. The concentration of each component in the Factor Mix is summarized in Table A2-8.

Different variations of the commercial and in-house system were combined to test specific components of the in-house system. To validate the in-house energy solution, Solution A in the commercial kit was replaced with 6.25 µL of the energy solution. To isolate specific components of the energy solution that were non-functional, a "poisoning test" was conducted, where each component of the in-house energy solution was added in 2-fold excess to the PURExpress® kit. To assess transcription only, the components required for transcription were combined, including 10 µL of Solution A or 6.25 µL of the Energy Solution, T7 RNAP, PPiase, 250 ng/µL of template DNA, and 0.5 µL of RNase Inhibitor (RiboLock, Thermo Fisher, E00381) with milliQ H_2O for a final reaction volume of 25 µL. Two different reaction concentrations based on reports from Shimizu *et al.* and Lavickova *et al.* were tested for T7 RNAP (0.77 µM and 0.1 µM) and PPiase (0.45 µM and 0.05 µM), respectively. The PURExpress® ΔRF123 kit (New England Biolabs, E6850S) was used to test RF1, RF2, and RF3, the PURExpress®Δ tRNA/aa kit (New England Biolabs, E6840S) was used to test the tRNA and amino acids, and the PURExpress® ΔRibosome kit (New England Biolabs, E3313S) was used to test the ribosomes. The user manuals for the commercial kits were followed, similar to the reaction conditions described above.

Successful *in vitro* translation was validated by measuring fluorescence of the reporter protein EYFP or by SDS-PAGE analysis of the control protein DHFR, which is provided in the commercial kit. *In vitro* transcription was confirmed by measuring the fluorescence of Spinach RNA, following the addition of the fluorophore 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), or by urea PAGE analysis.

2.3.8 FLUORESCENCE MEASURMENTS

A QuantaMaster Fluorimeter (Photon Technology International (Canada) Inc) was used to measure the emission spectra of the produced fluorescent proteins when excited at a specific wavelength. 25 µL of the *in vitro* protein synthesis reactions were diluted to a final volume of 150 µL with $TAKM_7$ buffer and placed in a quartz cuvette for analysis. To analyze reporter protein fluorescence, EYFP was excited at 513 nm and emission scanned between 525-625 nm (λ_{max} = 527 nm). To analyze Spinach RNA fluorescence, 20 µM of the fluorophore DFHBI was added following the 16-hour reaction incubation, which binds to the transcribed Spinach RNA. Spinach

RNA:DFHBI was excited at 450 nm and emission scanned between 465-565nm (λ_{max} = 498 nm). Prior to analysis, the negative control was subtracted from each emission spectra. Reproducibility of the emission spectra was assessed by performing multiple excitation and emission scans, resulting in nearly identical emission profiles (Figure A2-1).

An alternative method to analyze EYFP fluorescence was tested, providing a high throughput approach for measuring protein synthesis that was comparable to the measurements in the fluorimeter (Figure A2-2). 25 µL reactions were diluted with 125 µL of $TAKM_7$, added to a black 96-well plate, and imaged using the Amersham™Typhoon™5 (GE Healthcare). Densitometry analysis was performed using ImageJ software to measure and compare the fluorescent intensity between samples (*206*).

2.3.9 COST ANALYSIS

The cost to prepare the in-house *in vitro* reconstituted TX-TL system was calculated and compared to the four commercially available PURExpress® systems, as well as to previously reported *in vitro* translation systems (*71, 72*). The cost analysis was divided into three main sections: Energy Solution, Factor Mix and Ribosomes. Cost per reaction was calculated in Canadian dollars (CAD) for a standard 25 µL and for a 1 µL reaction volume. To produce the system in-house, a laboratory was assumed to be equipped with the infrastructure required for cell culture, protein expression and protein purification. The described ribosome purification does require specialized equipment (e.g., ultracentrifuge and zonal rotor), but alternative, simpler protocols are available (*72, 210, 212*). Cost estimates were made for lab supplies and consumables (e.g., pipette tips, syringe filters, microfuge tubes, centrifuge tubes, etc.). Reusable reagents and components following proper cleaning or regeneration was taken into consideration. Cost to prepare buffers was calculated based on buffer compositions. A value for graduate student work was included, based on current pay scales.

Preparation of the energy solution was assumed to require one day. A stock of each component was individually made and combined to form the final solution (summarized in Table S1). In total, a 2 mL solution was prepared, which is equivalent to 320 25 µL reactions, and cost $0.40 per 25 µL reaction and $0.016 per µL reaction (Table A2-11). Preparing a larger volume

would result in further cost savings. Cost per µL is comparable to the energy solution reported in Lavickova *et al.* at $0.019 USD or $0.023 CAD when using an exchange rate of 1.2 (as of June 10, 2021) (*72*).

The factor mix was composed of individually purified proteins, except CK which was purchased commercially (Table S6). To determine the cost of the TX-TL factor mix, cost per purification was first determined (Table A2-12). The cost estimate for size exclusion chromatography was based on FPLC pricing at the SynBridge core facility, University of Lethbridge, plus cost of the appropriate resin and column. Cost per purification was calculated at $303.54 per protein, assuming four purifications per week. Cost per reaction was calculated based on the cost per purification, the amount of protein produced, and the final protein concentration in the reaction, totalling $2.98 per 25 µL reaction and $0.12 per µL (Table A2-13). Note, there was a large range in protein yield, which is dependent on a variety of factors including the gene or protein sequence and the amount of cells grown and harvested. Optimizing the purification, including the use of a larger cell culture volume (from 1 L to 2 L), would reduce the cost per reaction. In total, enough protein was produced for at least 332 25 µL reactions (which was limited by EF-Tu), while in some cases protein was produced for 80000 25 µL reactions (e.g., T7 RNAP).

The ribosome purification results in the isolation of highly pure complexes. To achieve this level of purity multiple sucrose cushion ultracentrifugation steps followed by a sucrose gradient ultracentrifugation step was required to isolate the 70S complex over an 11-day period. Although this process is more time intensive compared to methods reported by Lavickova *et al*., there is a 16-fold increase in ribosomes produced, corresponding to approximately 120000 pmoles of ribosomes, which is enough for 750 25 µL reactions and cost $0.42, or $0.017 per µL reaction (Table A2-14). This process also allows for 30S and 50S fractions to be isolated, and a supernatant containing aaRS's, if desired.

The cost per reaction for each component was combined, totalling $0.18 per µL for the in-house TX-TL system. To provide an estimate of protein produced per cost of the system, the normalized EYFP expression levels comparing the in-house TX-TL system to the PURExpress® system was included.

2.4 RESULTS

In-house preparation of an *in vitro* reconstituted TX-TL system is a laborious, time consuming, and difficult process. It was our goal to identify common challenges encountered during this process and provide the corresponding guidelines or tools for improvement. Preparation of the in-house TX-TL system was divided into three parts: 1) energy solution, 2) TX-TL factors, referred to as the factor mix, and 3) ribosomes (Figure 2-1). Each component was individually characterized and tested using a variety of different tools, followed by complete reconstitution and comparison to the commercially available PURExpress® system. The *in vitro* reconstituted TX-TL system represents the minimal system for RNA and protein production outside of a cell. Therefore, purity of each component was an important consideration. Our findings and recommendations from this study build towards the production of a highly functional, cost-effective, easy to obtain, and modular *in vitro* TX-TL system.

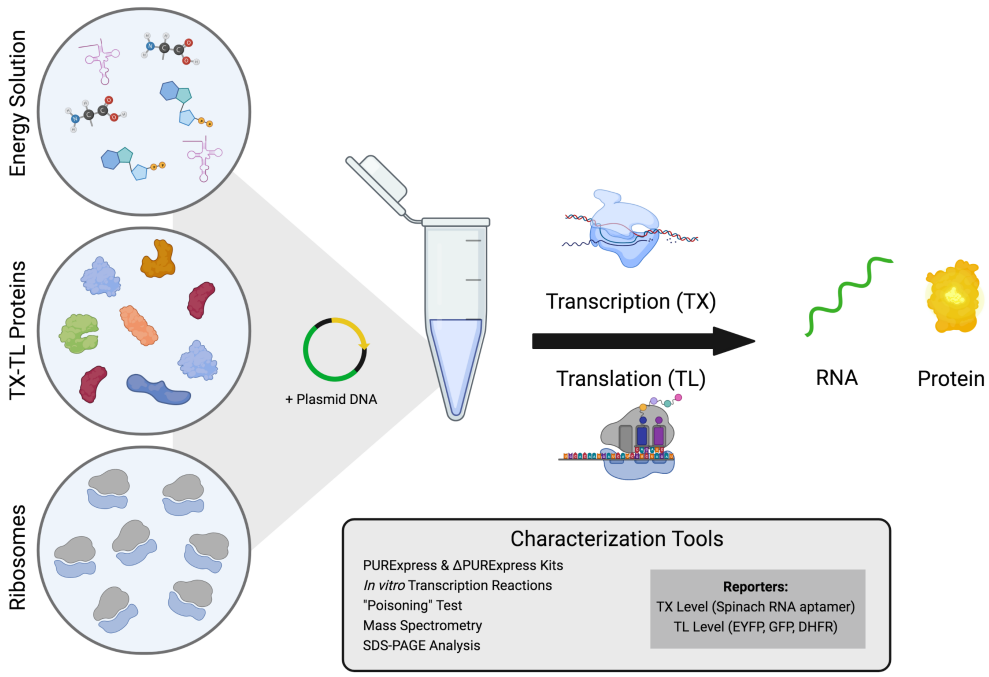


Figure 2-1: Components of an *in vitro* reconstituted TX-TL system.

In vitro TX-TL systems consists of an energy solution, individually purified TX-TL proteins, and purified ribosomes. Following addition of plasmid DNA, RNA and protein are produced. A variety

of characterization tools were employed to validate individual components and *in vitro* protein synthesis. Image was created in BioRender.

2.4.1 IN-HOUSE ENERGY SOLUTION VALIDATION

The in-house energy solution was prepared following methods described by Shimizu and Ueda (2010), with slight modifications (*210*). The energy solution is an important component of the *in vitro* TX-TL system as it supplies key substrates for protein synthesis, and for energy regeneration. Specifically, it is composed of amino acids, tRNA, NTPs, magnesium acetate, potassium glutamate, creatine phosphate, folinic acid, spermidine, and DTT (Table A2-1). Functionality of the energy solution was tested by combining with Solution B from the PURExpress® system and template DNA for the reporter protein EYFP. In comparison to its commercial equivalent (Solution A), the in-house energy solution was initially non-functional, observed by the absence of EYFP autofluorescence.

To identify which component of the energy solution was functional or non-functional, a systematic approach was followed. First, the amino acids and tRNA were validated using the PURExpress® ΔtRNA/aa kit. Using EYFP as the reporter protein, the in-house prepared amino acids and tRNA were comparable to amino acids and tRNA provided in the commercial kit, observed by similar fluorescence levels (Figure A2-3). Next, a “poisoning test” was conducted where each in-house component was supplemented to the PURExpress® system to identify if a specific component was toxic to the reaction (Figure A2-4). A reduction in protein synthesis was observed when supplementing the PURExpress® reaction with ATP, UTP, and creatine phosphate, while folinic acid prepared in a TAK-based buffer was slightly better than folinic acid prepared in a HCl-based buffer. Preparing new creatine phosphate resulted in an improved fluorescent signal (Figure A2-4), highlighting the importance of preparing fresh components.

NTPs were prepared following in-house methods used for setting up IVT reactions, which consisted of dissolving ATP, CTP, and UTP in a magnesium acetate solution, dissolving GTP in water, and adjusting the pH to 7.5. Alternatively, preparing all of the NTPs in water and adjusting the pH to 7.0 significantly improved the functionality of the energy solution, highlighting the

importance of an optimal magnesium concentration, which is consistent with previous reports (Figure A2-5) (*76*). With these improvements incorporated, the final prepared energy solution was comparable to Solution A, demonstrating similar EYFP fluorescence (Figure 2-2A). Depending on the batch of PURExpress® used, slight variations were observed (Figure A2-6). For example, in some cases, the in-house energy solution resulted in higher fluorescence levels compared to the commercial system. The differences in EYFP autofluorescence may reflect the negative impact of freeze thaw cycles on Solution A and B, which was also observed for the in-house energy solution (Figure A2-7), or as a result of batch-to-batch variation resulting from slight differences between system preparations.

2.4.2 IN-HOUSE FACTOR MIX VALIDATION

The factor mix consists of 36 proteins required for TX-TL (38 purifications total), including EF-Tu, EF-Ts, EF-G, IF1, IF2, IF3, RF1, RF2, RF3, RRF, aaRS's, MTF, energy regeneration components (MK, NDK, CK, PPiase), and T7 RNAP. Each protein was expressed in *E. coli* and purified by nickel affinity chromatography followed by size exclusion chromatography to ensure high protein purity (protein expression and purification details are summarized in section Table A2-9).

To improve recombinant protein expression in *E. coli*, each TX-TL gene was codon optimized. The genetic constructs were designed following the BioBrick assembly standard and included a T7 promoter, medium strength RBS, and T7 transcriptional terminator. The BioBrick standard was chosen to facilitate future assembly of multiple cistrons towards simplifying the number of plasmids required. Additionally, the use of consistent genetic elements provided a baseline expression level, upon which promoter and RBS strength can be adjusted to fine tune protein expression in the future. Each gene was hexahis-tagged on the N- or C-terminus, depending on previous reports for affinity purification (*69*) (Figure 2-2B).

Protein purity was confirmed following mass spectrometry, gel analysis, and spectrophotometer analysis. 28 of the proteins were >90% pure, 5 proteins were >80% pure, and 3 proteins were >70% pure (Table A2-8). Mass spectrometry analysis of NDK indicated a high level of tryptophan tRNA synthetase (TrpRS) contamination. When analyzed by SDS-PAGE a lower level

of contamination was determined, with NDK accounting for approximately 80% of the protein sample (Figure A2-8). Based on the gel analysis and given that additional TrpRS was not expected to have a negative effect on system performance, in-house purified NDK was included in the factor mix. In the case of CK, only 50% of the sample was identified as CK by mass spectrometry. CK plays an important role in the *in vitro* TX-TL system regenerating ATP. Due to its functional importance for energy regeneration, commercial CK was purchased and included in place of the in-house purified CK. Common contaminants identified from the individual mass spectrometry analysis are summarized in Table A2-10. The majority of identified proteins are considered to commonly co-purify during nickel affinity chromatography (*213*), in line with previous reports (*71, 72*), or are a part of the translation machinery (TX-TL proteins or ribosomal proteins). RNA and DNA contamination were assessed by measuring absorbance at 260nm and 280nm. An acceptable ratio (A260/A280) of approximately 0.6 was identified for the majority of proteins. A ratio greater than 1 was determined for AlaRS and RF1, indicating nucleic acid contamination, and suggesting an additional purification step, such as ion exchange, may be required for select proteins to obtain high protein purity.

RF2 was on the lower end of acceptable purity (~72%), while RF1 had a high level of nucleic acid contamination. To confirm that contaminating proteins and/or nucleic acid contamination had only a minimal effect on the *in vitro* TX-TL system, the in-house purified factors were tested in the PURExpress® ΔRF123 kit. Measuring EYFP reporter fluorescence showed in-house purified RFs were comparable to the commercial RFs (Figure A2-9). T7 RNAP is responsible for transcribing template DNA to mRNA. Due to its pivotal role in gene expression, T7 RNAP was individually validated and compared to a previously purified and functional T7 RNAP (control T7 RNAP). Two approaches were used to test the in-house T7 RNAP by measuring transcription of Spinach RNA: 1) in a standard IVT reaction and 2) in the context of the *in vitro* reconstituted TX-TL system. For the IVT reactions, urea PAGE analysis showed comparable RNA levels for both the control and test T7 RNAP (Figure A2-10). In the *in vitro* reconstituted TX-TL conditions, which used the in-house energy solution, Spinach RNA fluorescence was detected following the addition of DFHBI and concluded successful transcription. Increasing the concentration of T7 RNAP increased

Spinach RNA:DFHBI fluorescence (Figure A2-10). Overall, under the *in vitro* reconstituted TX-TL reaction conditions, more RNA was produced when compared to the IVT reaction conditions. In conclusion the transcription component of the TX-TL mix was functional.

The TX-TL components were combined following similar concentrations as described in the homemade PURE preparation by Lavickova *et al.* (2019), which in their study resulted in ~56% of the PURE protein synthesis activity (*72*). Overall, the in-house factor mix in combination with the in-house energy solution and commercial ribosomes resulted in 37.8% protein synthesis activity compared to the commercial PURExpress® system based on EYFP autofluorescence (Figure 2-2D). Although the system was capable of protein synthesis, the reduced expression level indicates further troubleshooting is required to improve protein yield. Furthermore, when analyzed on an SDS-PAGE the in-house factor mix composition differs from the proprietary commercial factor mix, suggesting optimized component concentrations will improve yields (Figure 2-2B).

2.4.3 IN-HOUSE RIBOSOME VALIDATION

Ribosomes were isolated from *E. coli* MRE 600 cells. A multi-step purification was employed, including multiple sucrose cushion ultracentrifugation steps followed by sucrose gradient ultracentrifugation to isolate highly purified 70S ribosomes (Figure 2-2C). The PURExpress® Δ Ribosome kit was used to compare the in-house prepared ribosomes to commercial ribosomes. Overall, the in-house ribosomes were capable of protein synthesis, and showed 60% EYFP fluorescence compared to the complete commercial system (Figure 2-2D). Due to proprietary reasons, it is unclear what differences between the in-house and commercial ribosomes may account for the observed variation in fluorescence levels. For example, cell strain and purification method will influence ribosome composition. It may be hypothesized that the in-house ribosomes undergo a more stringent isolation process compared to the commercial preparation, removing associated factors that are important for protein synthesis (*expanded on in Chapter 3*). Therefore, differences in purification method will have an effect on the final performance of the system.

2.4.4 FUNCTIONAL IN-HOUSE RECONSTITUTED TX-TL SYSTEM

The in-house prepared energy solution, factor mix, and ribosomes were each functional when individually tested. In combination, the in-house reconstituted TX-TL system was also functional and capable of protein synthesis. In comparison to the PURExpress® system and considering the results from the individual component tests, a lower level of EYFP fluorescence was expected and estimated to be ~23% of the commercial system. Surprisingly, the in-house reconstituted TX-TL system yielded 4% of the fluorescence achieved using the commercial system (Figure 2-2D), indicating more improvements are needed to improve protein yield.

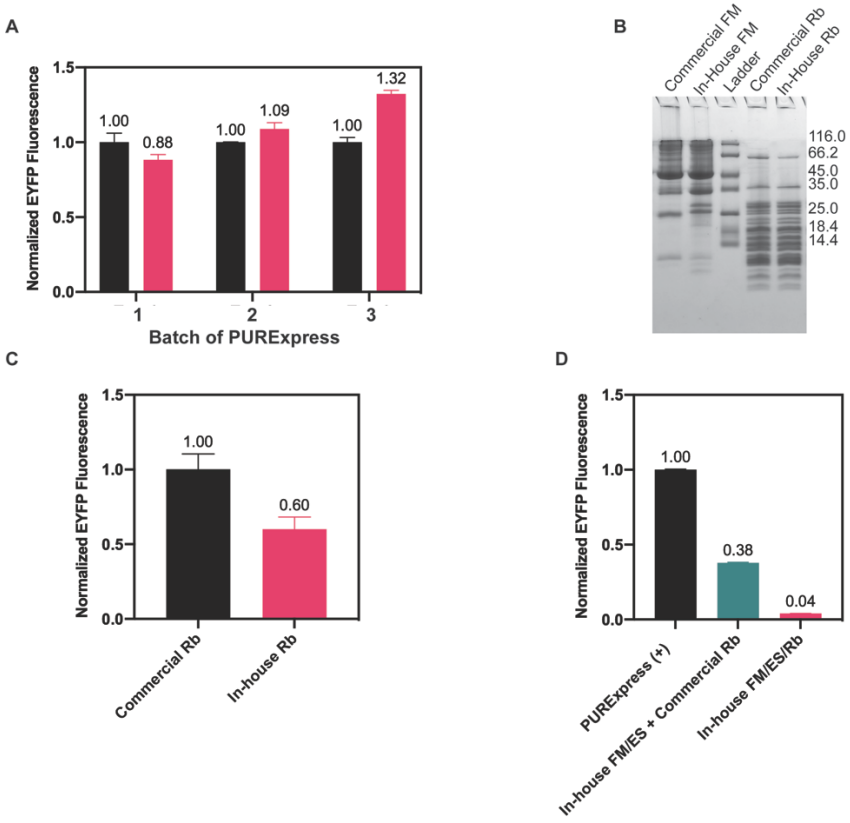


Figure 2-2: In-house prepared *in vitro* reconstituted TX-TL components are capable of driving protein synthesis.

(A) In-house energy solution was functional and comparable to Solution A from PURExpress®, demonstrated by similar EYFP fluorescence levels. Three tests were performed using three different batches of the commercial system. (B) SDS-PAGE analysis comparing the in-house and

commercial factor mix, and the in-house and commercial ribosomes. (C) In-house ribosomes were functional, demonstrated by observed EYFP fluorescence. Tests were conducted using the PURExpress® ΔRibosome kit, performed in triplicate. (D) In-house factor mix was functional, when combined with the in-house energy solution and commercial ribosomes. Complete reconstitution of the in-house prepared components (factor mix, energy solution and ribosomes) was capable of protein synthesis, demonstrated by EYFP fluorescence. Protein yield was low and requires further optimization. Three excitation and emission scans were performed. EYFP was excited at 513 nm and emission scanned between 525-625 nm (λ_{max} = 527 nm). Negative control was subtracted from the emission spectra.

2.4.5 COST ANALYSIS OF AN IN-HOUSE RECONSTITUTED TX-TL SYSTEM

Through a detailed cost analysis, the in-house TX-TL system was $0.15 CAD per µL of reaction, which is approximately one tenth of the cost of the PURExpress® system at $1.51 CAD per µL, demonstrating the value of large-scale in-house production (Figure 2-3A). The delta versions of the commercial systems are more expensive per reaction, while the in-house system, in addition to being cheaper to produce, is more flexible regarding custom composition (i.e. removal of specific components, increased or decreased concentration of specific components). Similar cost estimates were given for the previously reported TraMOS and OnePot, while the in-house TX-TL was more cost-effective in comparison to the corresponding PURE-lab and HomeMade PURE. Theoretically, similar processes were followed to produce the in-house TX-TL, PURE-lab, and HomeMade PURE. However, limited information detailing the associated cost analyses hindered direct comparison between the three systems. When normalized per protein, the in-house system costs $3.80 per yield, a 2.5-fold increase compared to the PURExpress® system (Figure 2-3B). To further reduce the cost per yield and reach similar protein synthesis activity achieved with the PURExpress® system, a small improvement of ~6% in protein yield is required. Further increases in yield would produce a more cost-effective system, in addition to the already present benefit of customizability.

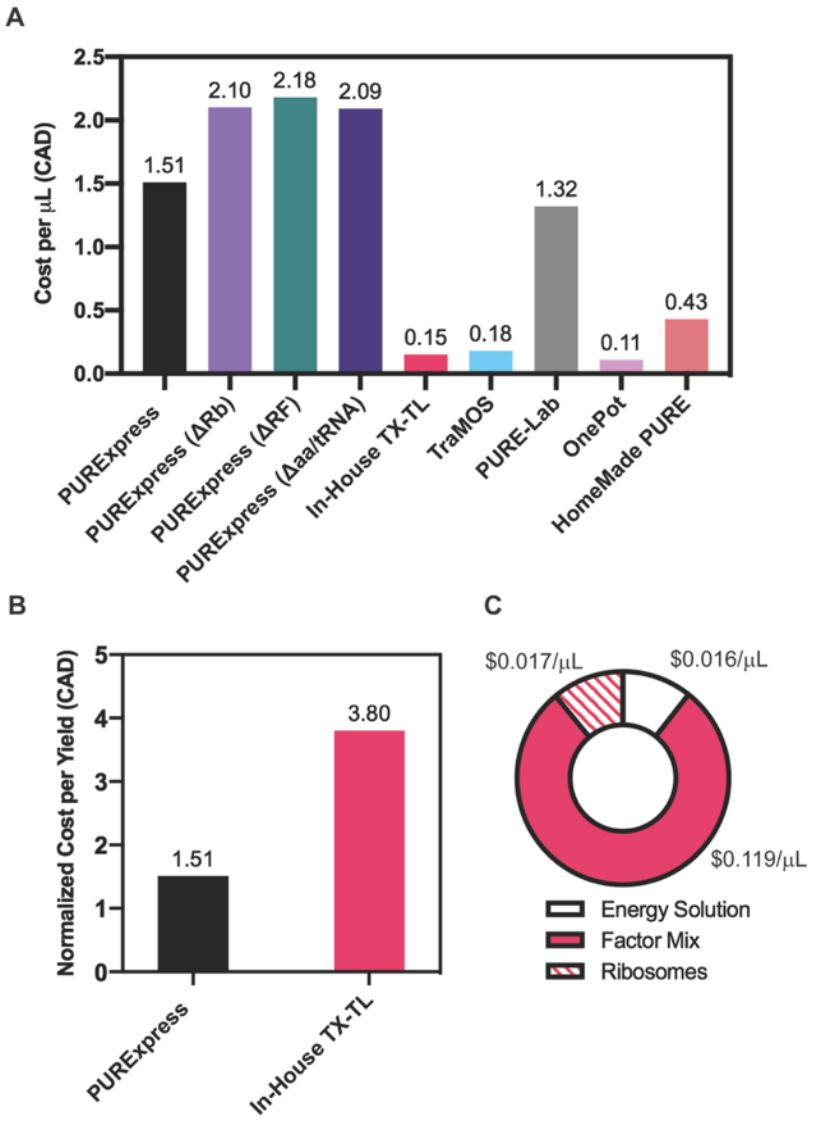


Figure 2-3: Preparing *in vitro* reconstituted TX-TL components in-house provides a cost-effective approach to achieve cell-free protein synthesis.

(A) Cost comparison of in-house TX-TL system to commercially available and previously published *in vitro* reconstituted TX-TL systems. Reported as cost per µL of reaction. Cost to prepare TraMOS and PURE-Lab were reported in Villarreal *et al.* (*71*); OnePot and HomeMade PURE were reported in Lavickova *et al.* (*72*) (B) Normalized reaction cost per yield of protein. Estimated based on normalized EYFP fluorescence when comparing the in-house TX-TL system to PURExpress®. (C) Cost breakdown for each component of the in-house TX-TL system per µL of reaction.

2.5 DISCUSSION

2.5.1 COST BENEFIT OF PRODUCING AN *IN VITRO* RECONSTITUTED TX-TL SYSTEM IN-HOUSE

Overall, the in-house TX-TL system was demonstrated to be functional, and when produced in-house has the added benefit of increased control. Such features provide the flexibility to customize the system for a desired purpose or application. Furthermore, in-house production provides access to larger reaction volumes. To be cost effective large amounts of energy solution, factor mix, and ribosomes were prepared. For example, the energy solution can be easily scaled-up and produced in bulk, further reducing the cost per reaction. Additionally, by preparing a concentrated stock of each individual component, customized energy solutions can be made. In total, the energy solution cost $0.016 CAD per µL reaction, and was comparable to reports by Lavickova *et al.* (2019), which reported $0.019 USD per µL reaction (this is equivalent to $0.023 CAD per µL reaction when using an exchange rate of 1.2). Directly comparing the cost of each component per reaction revealed the in-house preparation of NTPs to be more cost effective when purchasing as a salt compared to purchasing in solution. The energy solution cannot be purchased separately, unless specifically requested from NEB. Due to this limited access, it is very useful to have the tools available at hand to prepare a highly functional and cost-effective energy solution in the lab.

The most expensive component of the in-house TX-TL system was the factor mix, which cost $0.119 CAD per µL reaction. To purify each protein a standard cost was determined. Each purification yielded a different amount of protein corresponding to enough protein to support a range of reactions from a few hundred to a few hundred thousand. Therefore, the cost of protein per reaction was highly variable. By improving the overall yield per purification, it is possible to lower the cost per reaction. Methods to achieve this include optimizing the expression and purification of select proteins that are limiting (e.g., EF-Tu).

The cost of in-house ribosomes per µL reaction is ~100-fold cheaper compared to purchasing commercial ribosomes. One ribosome purification produces on average 120 000 picomoles of 70S ribosomes, which corresponds to 50 000 µL reactions. In comparison to ribosome isolation methods described in Lavickova *et al.* (*72*), this is a 16-fold increase in yield. At different steps of the ribosome purification process fractions corresponding to the 30S and 50S subunits can also be isolated, as well as a supernatant containing the aaRS's, adding another advantage to this method.

In summary, the complete in-house TX-TL system cost $0.15 CAD per µL reaction. Improvements to each step of the preparation process, including increasing the amount of protein or ribosomes produced per purification or increasing the volume of energy solution, to reduce the cost per reaction. Furthermore, improvements to the system functionality regarding protein yield will further improve cost efficiency.

2.5.2 PREPARATION GUIDELINES

The composition of the *in vitro* reconstituted TX-TL system consists of the minimal components needed to produce RNA and protein outside of a living cell. Therefore, the purity of each component was important to truly represent the minimal system. To this end, a stringent purification protocol was followed to produce highly pure 70S ribosomes, specific components of the energy solution were filter sterilized, and a two-step purification method was employed to individually purify each TX-TL protein. Furthermore, the purity of each protein was assessed by mass spectrometry, and an analysis of the contaminating or co-purifying proteins was provided. Continuing to assess protein purity in this manner will help to inform the overall quality of the factor mix and contribute to understanding any variations in system performance.

A major challenge in preparing the cell-free system was the individual expression and purification of 38 proteins. To simplify this process a standard expression and purification method was followed. A summary of observations is given in Table A2-9, providing a reference at each step of the process. For example, some proteins expressed better than others, indicating that a larger culture volume may be required to increase cell mass to obtain higher protein yields. It was also common to observe two peaks on the SEC chromatogram for the aaRS's. This was likely the

result from a reaction of the protein binding to cellular tRNA, as an increase in absorbance at 250 nm was observed. Overall, this serves as an example of the challenges encountered when using a single, standardized purification strategy for the purification of a wide range of different proteins as represented by the 38 components of the TX-TL system. Tables A2-8 and A2-9 provide important information, such as target protein concentrations and expected observations, to simplify and benchmark TX-TL protein expression and purification.

One useful tool, implemented throughout the troubleshooting process was a “poisoning test”, where in-house prepared components were added to the commercial system to identify easily and quickly if the component resulted in a detrimental effect. Results from this type of test were not considered to be conclusive, as it is possible that increased concentrations of select components could have a negative effect on translation, but instead provided a starting point to make improvements. Through this approach, we identified issues with creatine phosphate and NTPs in the energy solution. Other important tips and considerations for preparing the energy solution include adjusting the final pH of the system to 7.0 and preparing concentrated stocks to simplify sub-stock preparation. Lastly, the use of a validation construct was valuable to individually characterize transcription and translation of the cell-free system. By measuring Spinach RNA levels, components required for transcription were validated prior to assessing translation. This provided an additional tool to debug specific aspects of cell-free protein synthesis gene expression. Furthermore, the impact of transcription on translation is unknown. Both components of the biomolecular machinery share the energy pool (ATP and GTP). Therefore, transcription levels, and the corresponding depletion of ATP and GTP, may have an effect on translational output. There is potential to expand the Spinach RNA reporter to provide additional information on the amount of RNA produced, which can assist in understanding how these two processes impact each other.

2.6 CONCLUSION

In summary, a purified *in vitro* reconstituted TX-TL system was prepared and shown to be capable of DNA template directed protein synthesis. The in-house energy solution was comparable, and in some cases better, than its commercial equivalent, while the in-house ribosomes and factor

mix were functional but to a lesser extent. Although a relatively low protein yield was observed, the system is cost-effective to produce in-house and provides benefits of increased control over the system composition. Modeled after previously published work, this study provides the first detailed characterization of the purified TX-TL components and troubleshooting tips to guide in-house preparation, including information about each individual protein purification, how to prepare a highly functional energy solution, and the utility of a validation tool to test TX-TL. Through improved methods, we contribute to improving accessibility of cell-free systems, facilitating its application in biotechnology development.

CHAPTER 3 - PROTEOMIC ANALYSIS REVEALS COMPOSITIONAL INSIGHTS OF AN *IN VITRO* RECONSTITUTED TX-TL SYSTEM

3.1 PREFACE

Chapter 3 describes the proteomic analysis of an *in vitro* reconstituted TX-TL system. The study is presented in manuscript form for submission to *BMC Genomic Data*, an open-access journal that promotes data transparency. By providing a complete picture of the protein composition and ribosomal protein stoichiometry, quality of the protein component can be monitored between batches and across different types of cell-free systems. I report the identification of contaminating proteins that may negatively impact translation, as well as other factors that may have a beneficial effect, providing valuable information for the optimization of cell-free protein expression. Overall, the reported proteomic analysis provides a useful tool towards deciphering key factors of the translation machinery for improved cell-free protein synthesis and for understanding translation mechanisms.

Contributions of Authors: The study was planned by Dr. Han-Joachim Wieden and myself. Mass spectrometry was performed by the University of Lethbridge proteomics facility (Fan Mo), while I conducted subsequent data analysis. I wrote the manuscript.

3.2 INTRODUCTION

In vitro TX-TL systems provide an alternative approach to cell-based, *in vivo* recombinant protein expression. Also referred to as cell-free systems, *in vitro* TX-TL systems are free of the burden to maintain cellular homeostasis and are open, unrestricted by the cellular wall and membrane. Such features provide a design-friendly and engineerable platform for the execution of a desired function and enable *in vitro* TX-TL systems to be used for a variety of applications (*35, 46, 181*). For example, cell-free systems are capable of carrying out functions that are difficult or not possible to implement *in vivo,* including for the expression of toxic compounds with therapeutic or industrial relevance that would be detrimental to a cell if expressed *in vivo*, or for the incorporation

of non-canonical amino acids expanding the realm of possible protein functions (*46, 214*). Cell-free systems also provide a foundation for constructing artificial life and for creating synthetic minimal cells (*91*), as well as aid in elucidating fundamental processes regarding the molecular mechanism of protein synthesis .

Cell-free systems derived from *E. coli* are the most commonly used and are available in two forms: 1) as cell lysates, which typically refers to S30 extracts (*35*), or 2) as purified reconstituted components, which consists of the minimal biomolecular machinery required for transcription and translation (*68*). In the latter case, there is a trade-off between having a defined and controllable environment for gene expression and the time and effort required to prepare the system in-house or the high cost and limited modularity associated with purchasing a commercial system (e.g., PURExpress®). On the other hand, cell lysates are more cost effective and easier to produce but due to the complexity of the composition are limited with respect to customization.

Due to the potential applications and uses of *in vitro* TX-TL systems, research has focused on the optimization of protein expression, because these systems generally do not reach the same performance levels that are achieved *in vivo*. Cell-free protein synthesis is limited by low protein yield and impaired by the expression of truncated or misfolded products (*76, 81, 82*). This is especially true in the case of purified *in vitro* reconstituted systems, where protein yield is approximately a fourth of what is produced in cell lysates (*215, 216*). Ribosomes, and ribosome-dependent processes, have been identified as critical factors contributing to poor gene expression, including issues with ribosome initiation, stalling, and recycling (*76, 81-83*). Overall, these limitations impact potential biomanufacturing capabilities and scale-up, as well as the ability to produce the components required for a self-regenerating minimal cell (*217*).

Towards improving the functionality of *in vitro* reconstituted TX-TL systems, previous studies have aimed to optimize component concentrations and to supplement the system with different factors to improve translation (*74, 76, 77, 81, 218*). The main protein component of purified *in vitro* reconstituted systems typically consists of ribosomes and 36 transcription and translation factors. This is significantly reduced from the approximately 900 proteins identified in cell lysates (*59*) and over 4000 genes in the *E. coli* genome (*219*). Kazuta *et al*. (2008) demonstrated the complex

protein-protein interaction network by testing the effect of individual *E. coli* proteins on the *in vitro* expression of GFP (*77*). Of the 4194 proteins tested, 12% were shown to interact, either positively or negatively, with the *in vitro* reconstituted TX-TL machinery. Narrowing down which of these proteins are most optimal for improving *in vitro* gene expression, while also maintaining a minimal reconstituted system, is a challenging task. Furthermore, it is often difficult to produce and troubleshoot cell-free expression systems made in-house. Batch-to-batch variation, issues with shelf-life, and limited reaction lifespan contribute to observed differences in protein yield and add a layer of unpredictability regarding gene expression levels (*100*). Therefore, additional tools are required to aid in the optimization of *in vitro* reconstituted TX-TL systems and improve system performance.

Although purified *in vitro* reconstituted systems have a defined composition, we hypothesize that a proteomic analysis may reveal insights into the stoichiometry of important factors (i.e. ribosomes, and transcription and translation factors), and identify the presence or absence of other proteins that have a positive or negative effect on gene expression due to interactions with the minimal TX-TL components. This information would provide a benchmark of current systems and guide further improvements towards increasing protein yield, which is important for cell-free application in biotechnology development, and to improve our understanding of the underlying molecular mechanisms of protein synthesis.

We assessed the complete composition of a purified *in vitro* reconstituted TX-TL system by performing a proteomic analysis of commercially available and in-house prepared components, with an emphasis on ribosome composition, via liquid chromatography-tandem mass spectrometry (LC-MS/MS). To evaluate the protein composition, ribosome protein stoichiometry, presence of non-ribosomal factors, relative ratios of transcription and translation factors, and the presence of contaminating proteins was assessed. Comparisons to S30 lysate proteomic data, as well as to previous studies describing modifications to purified *in vitro* reconstituted systems, allowed for recommendations to be made regarding potential candidates to test further. Additionally, a proteomics approach provides a way to monitor system quality between batches and between different types of *in vitro* TX-TL systems.

3.3 METHODS

3.3.1 REAGENTS

Chemicals and reagents used in this study were obtained from BioBasic, unless otherwise specified.

3.3.2 PURIFICATION OF RIBOSOMES

Ribosomes were purified from *E. coli* MRE 600 following methods described in Rodnina *et al.* (1994) (*209*).

3.3.3 PREPARATION OF IN-HOUSE FACTOR MIX

Refer to methods in section 2.3.2.

3.3.4 RIBOSOME AND FACTOR MIX VALIDATION

Refer to methods in section 2.3.8. To test the effect of additional ribosomal protein S1 on gene expression, 30 pmoles of purified S1 was added to the reaction (see section 4.3.2 for detailed purification methods).

3.3.5 MASS SPECTROMETRY SAMPLE PREPARATION

Protein samples were prepared following an in-gel digestion method to denature proteins. Protein samples were resolved on a 12% SDS-PAGE, stained with Coomassie Blue R-250 for protein visualization, and excised from the gel. SDS-PAGE gel samples were first washed with H_2O and dehydrated with acetonitrile (ACN). Samples were then reduced with 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 at 56°C for 50 minutes and alkylated with 55 mM in 100 mM NH_4HCO_3 iodoacetamide for 20 minutes in a thermoshaker at 26°C, 1050 rpm. After two washes with 100 mM NH_4HCO_3, the samples were digested with 12.5 ng/ml trypsin solution with 41.7 mM NH_4HCO_3 and 4.17 mM $CaCl_2$ at 37°C overnight. Digested peptides were extracted with ACN, and dried. Then peptides were reconstituted in 5% FA.

3.3.6 LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS)

Data was acquired on a Thermo Scientific Orbitrap Fusion mass spectrometer coupled with a Thermo Scientific EASY-nLC 1000. 450 ng of reconstituted peptide samples (in triplicate) were loaded on a 0.075 mm x 500 mm Accucore 150 C18 with 2.6 µm particles at a flow rate of 200 nl/min with a 20 mm Acclaim PepMap 100 C18 trap column. Samples were separated on a short

gradient from 3% to 13% B (7 min), 13% to 44% B (50 min), 44% to 100%B (5 min) and 100% B (11 min), or long gradient from 3% to 13% B (15 min), 13% to 44% B (100 min), 44% to 100%B (10 min) and 100% B (10 min). Solvents contained A: 0.1% FA in water, and B: 0.1% FA in 80% ACN. MS/MS methods were programmed in the data dependent acquisition top speed mode, MS1 surveys were acquired in orbitrap and MS2 surveys were acquired in ion trap. For MS1 scans, the scan range was from 300 to 1500 m/z, with resolution at 120,000, and AGC target at $2x10^5$, 12 s exclusion duration. Ions with charge states 2+ to 7+ were subjected to MS/MS acquisition with HCD. For MS2 HCD scan at 1.2 s cycle time, the isolation window was 1.2 m/z, the collision energy was 30%, the AGC target was $1x10^4$, and the maximum injection time was set to 35 ms.

3.3.7 MASS SPECTROMETRY DATA PROCESSING AND ANALYSIS

Raw data were loaded into Maxquant 1.6.1.0, using default settings with iBAQ and match between run enabled, or Thermo Proteome Discoverer 2 with Precursor area quan IT HCD sequest HT percolator workflow, with default setting. To identify peptides, the reference data set UP000000625 *E. coli* strain K12 was used. For analysis of the transcription and translation components, the database was supplemented with protein sequences for CK, PPiase, and T7 RNAP, as the coding sequences are derived from organisms other than *E. coli.*

Data was filtered as described in Smits *et al.* (2013) (*207*). In brief, contaminants, reverse hits, and proteins with unique peptide counts less than 1 and/or with counts less than 2 were removed. Three methods, as described in the referenced literature, were used to analyze the data: 1) normalized iBAQ values (*208*), 2) peptide intensities (MS1) normalized by molecular weight (MS1/MW) (*220*), and 3) normalized EIC values (*221*). Statistical significance was determined using a multiple t-test (Holm-Sidak method), with α = 0.01 (** $p \leq 0.01$) or α = 0.05 (* $p \leq 0.05$) using GraphPad Prism software. Each row was analyzed individually, without assuming a consistent standard deviation (SD).

3.3.8 GENE ONTOLOGY ANALYSIS

To classify identified genes by gene function and protein class, gene ontology analysis was performed using the PANTHER (protein annotation through evolutionary relationship) classification system (http://www.pantherdb.org/).

3.4 RESULTS & DISCUSSION

3.4.1 OVERVIEW OF A LABEL-FREE PROTEOMICS APPROACH

Stable isotope labeling techniques have been extensively used for quantitative proteomic analysis by providing an internal standard or reference, but due to the complex and time-consuming sample preparation, expensive reagents required, large amounts of sample needed, and issues with labeling efficiencies, research on the much simpler label-free quantitative approach has expanded to overcome these challenges (*222*). Providing a faster and cheaper alternative, label-free quantitative proteomics measures changes in chromatographic ion intensities (i.e. peptide peak areas or peptide peak heights) or changes in MS/MS spectra (i.e. peptide peak intensities) allowing for the identification of protein relative abundance (*222*). Previous studies have demonstrated the utility and reasonable precision of label-free proteomics for providing important information on protein complex stoichiometry and protein relative abundance (*220, 223, 224*). Therefore, a label-free proteomics approach was used to characterize the composition of the purified *in vitro* reconstituted TX-TL components (Figure 3-1).

Protein relative abundance was primarily determined using the iBAQ method. The iBAQ value represents the sum of all peptide intensities for each protein divided by the number of theoretical peptides produced from a trypsin digestion (*208*). When normalized by the sum of all iBAQ values, protein relative abundance is inferred, allowing for comparisons between samples (*208*). For the assessment of ribosomal protein stoichiometry, two additional analysis methods were used as a comparison to the iBAQ approach. The first method normalizes the sum of MS, or peptide, intensities by the molecular weight, referred to as MS1 over MW, and has been used to evaluate protein stoichiometry of the 26S proteasome with good accuracy (*220, 223*). The second approach determines protein abundance by evaluating each protein's extracted ion chromatogram (EIC). Each protein EIC is normalized by the sum of all EIC's to provide information on relative abundance (*221*).

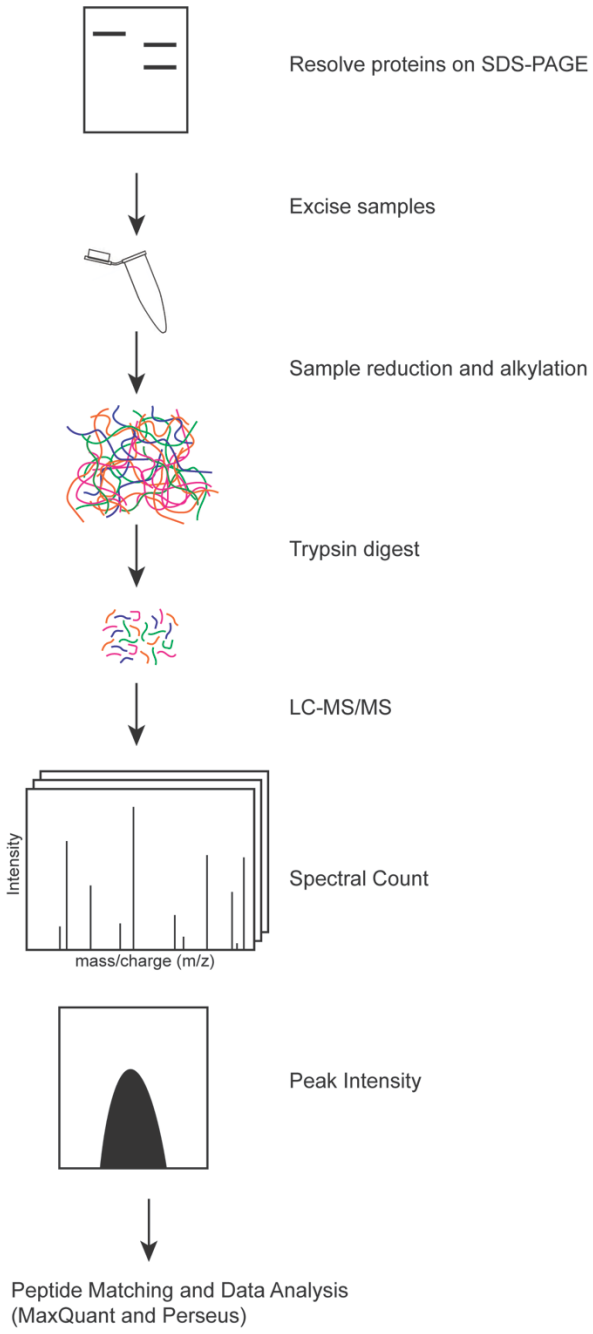


Figure 3-1: Analysis pipeline for label-free proteomics.

Samples for mass spectrometry are first resolved on an SDS-PAGE, followed by band excision, sample reduction, alkylation, and trypsin digestion. The sample composition is analyzed by LC-MS/MS, resulting in spectral count and peak intensity data. Software programs, such as MaxQuant and Perseus, are used for peptide matching and further downstream analysis of the data. Figure is adapted from Zhu *et al.* (2010) (*222*).

3.4.2 RIBOSOMAL PROTEIN STOICHIOMETRY

Ribosomes are macromolecular machines that play a pivotal role in gene expression, decoding mRNA and catalyzing peptide bond formation. Currently, ribosome performance and ribosome-associated processes are considered sub-optimal in cell-free expression systems, indicating a need to improve overall functionality (*76, 81-83*). To provide a baseline understanding of ribosome composition, commercial and in-house prepared ribosomes were analyzed by mass spectrometry to determine the presence and stoichiometry of ribosomal proteins.

For both the commercial and in-house ribosomes, all ribosomal proteins were detected with sequence coverage greater than 50% for the majority of proteins (Figure A3-1). L20 (13.5 kDa), L34 (5.8 kDa), L35 (7.3 kDa), and L36 (4.4 kDa) were identified with low (<50%) sequence coverage. These proteins are small in size, which sometimes can lead to difficulties in identifying them using bottom-up proteomics, if fewer tryptic peptides are produced. Additionally, if a small protein also has many trypsin cut sites, such as L36 (7 lysine and 5 arginine residues), the peptide size might be below the threshold of detection. Due to these reasons, L35 and L36 have previously not been detected in cell lysates (*60*). Overall, our approach was sensitive enough to detect all ribosomal proteins, indicating the reliability of our detection pipeline.

The expected ribosomal protein stoichiometry is one copy per ribosome (1:1), except for L7/L12 which is present in four copies per ribosome (4:1) in *E. coli* (*225, 226*). Although there are slight differences between the results obtained from the three evaluation methods (MS1 over MW, iBAQ, and EIC), a 1:1 stoichiometry was not observed (Figure 3-2). Instead, a large variation between ribosomal protein levels was identified. However, for each method, direct comparisons between the commercial and in-house ribosomes resulted in similar abundance levels for each ribosomal protein. This suggests that the deviation from the expected stoichiometry likely is the result of sample processing and instrument sensitivity (*227*).

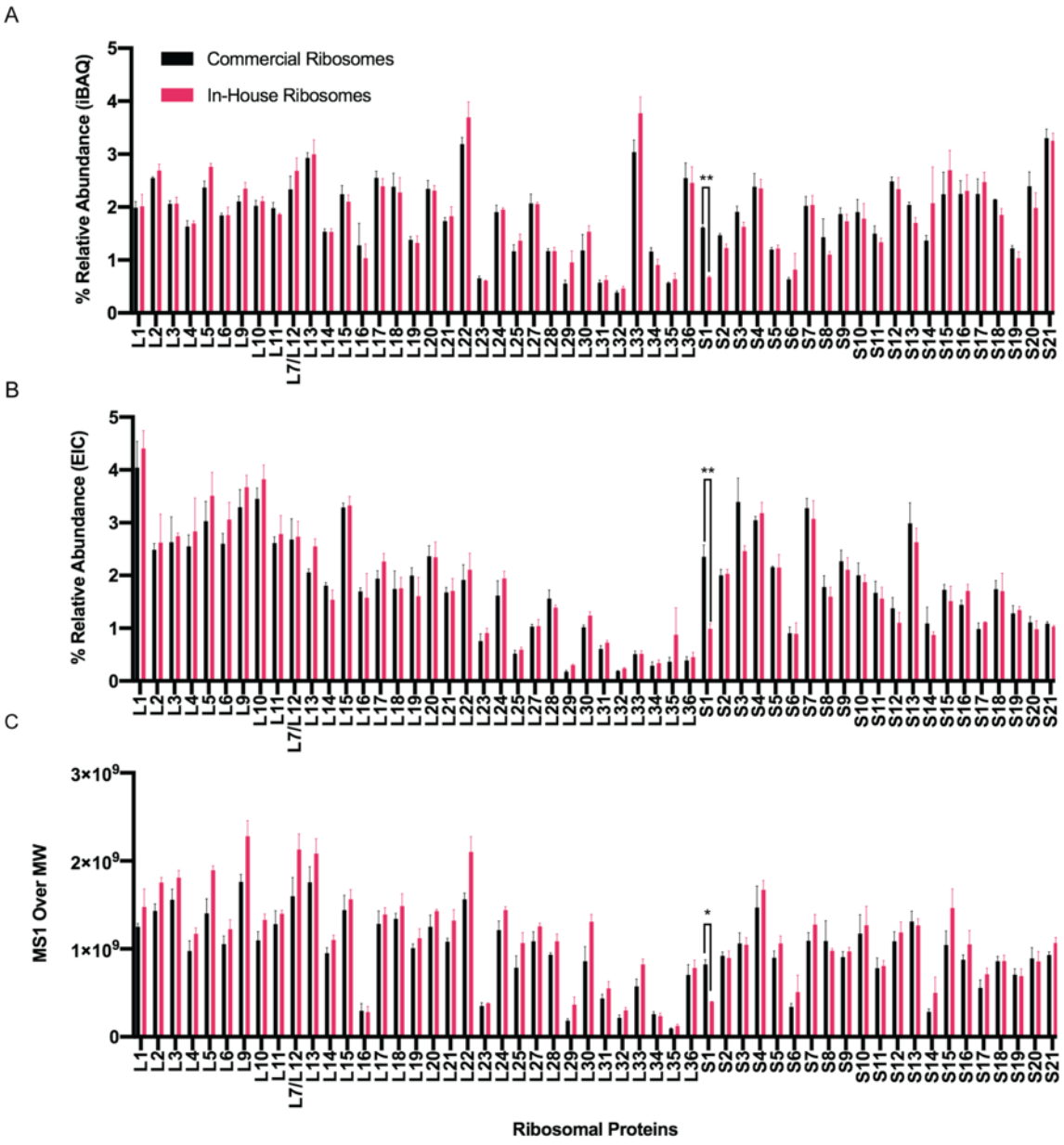


Figure 3-2: Ribosomal protein stoichiometry is comparable between the commercial and in-house preparations.

Ribosomal protein stoichiometry was evaluated using three methods to assess the commercial (black) and in-house (pink) ribosome composition: (A) Percent relative abundance determined using iBAQ values. (B) Percent relative abundance determined using EIC values. (C) MS1 values normalized by molecular weight (MW). Statistical significance was determined using a multiple t-test (Holm-Sidak method), with α = 0.01 (** p≤0.01) or α = 0.05 (*p ≤0.05), (n=3).

A remarkable exception to this observation was ribosomal protein S1, which did not show the correspondence in abundance levels between ribosomes and was present in statistically significant lower amounts in the in-house preparation compared to the commercial ribosomes. Supplementing the in-house ribosomes with purified S1 resulted in improved gene expression as reflected in an increase in EYFP fluorescence (Figure 3-3). Ribosomal protein S1 is known to loosely bind the 30S subunit, and therefore may be present in less than expected stoichiometric amounts following purification, further validating the sensitivity of our quantification pipeline (*228*). These results suggest differences in ribosome purification methods, resulting in lower levels of ribosomal protein S1 for the in-house preparation. Overall, the proteomic analysis provided a quality control tool to measure ribosomal protein abundance between different ribosome preparations and informed the addition of a specific factor to improve gene expression.

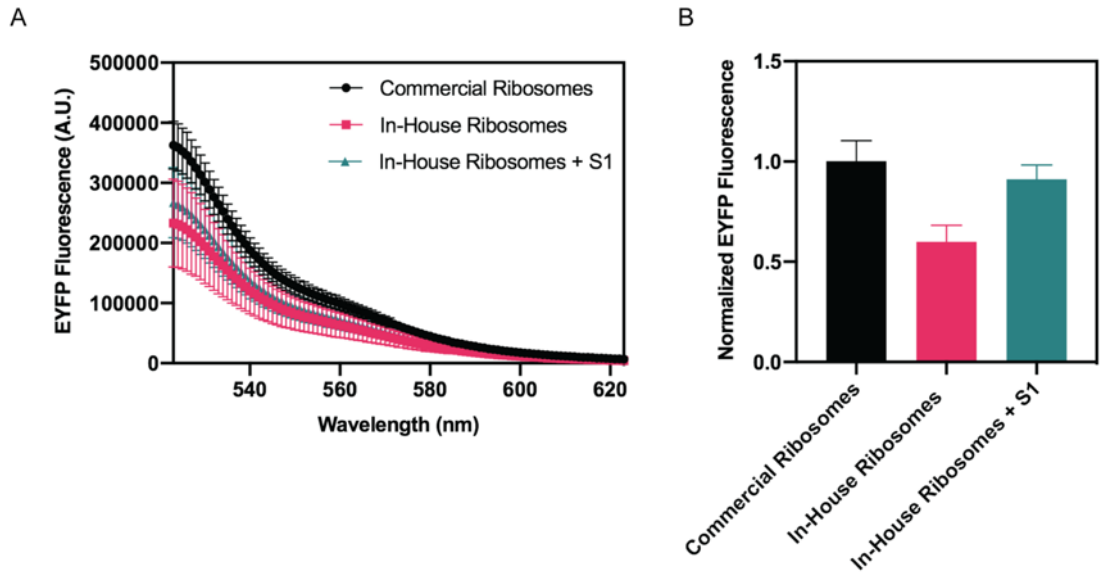


Figure 3-3: Addition of ribosomal protein S1 to the in-house ribosomes improved protein synthesis.

(A) EYFP emission spectra provides a measure of EYFP expressed from *in vitro* reconstituted cell-free reactions with commercial ribosomes (black), in-house ribosomes (pink), or in-house ribosomes with S1 (teal) (30 pmol added). EYFP excitation = 513 nm, λmax = 527 nm. (B) Fluorescence measurements normalized to the positive control (black) at the peak emission wavelength.

3.4.3 RELATIVE ABUNDANCE OF TX-TL FACTORS

In addition to ribosomes, TX-TL factors make up an important component of the purified *in vitro* TX-TL system, referred to as the factor mix (FM). Similar to the above analysis, the FM composition was characterized for a commercial and in-house mixture, as well as for a pre-mixed commercial solution consisting of FM and ribosomes (Rb). The in-house and commercial FM is composed of 38 individually purified proteins ranging in size from 8 – 108 kDa and varying in protein concentration. Some of the required factors for TX-TL were interestingly not detected. Specifically, PPiase (22.2kDa) was missing in all three cases (commercial FM, in-house FM, and commercial FM plus ribosomes); CK (44.5 kDa) and T7 RNAP (100.2 kDa) were not detected in the FM only cases; MK (25kDa) was not detected in the commercial systems, and TyrRS (48.9 kDa), MetRS (76.3 kDa), CysRS (53.5 kDa) and SerRS (49.8kDa) was also not present in the in-house factor mix. Protein concentration, protein size, and amino acid composition may impact the ability to be detected by mass spectrometry. Alternatively, these results may indicate an error in solution preparation and identify specific factors that require optimized protein concentration in the final mix.

To determine if protein abundance was similar for all three systems, the iBAQ values were normalized to EF-Tu, the most abundant protein in the expression system. This approach has been used to characterize cell lysates and compare protein abundance between samples (*61*). Significant differences in relative protein abundance were identified when comparing the commercial systems, with higher amounts of translation factors identified when ribosomes are included (Figure 3-4). These differences suggest that some factors co-purify with ribosomes, increasing their relative abundance in the final reaction mixture. Comparing between the commercial and in-house factor mixes revealed a statistically significant higher relative abundance for EF-Ts and TrpRS for the in-house mix, while for the other detected factors relative abundance levels were comparable.

Reported protein concentrations from previously published *in vitro* translation systems (*68, 72*), were also normalized to EF-Tu and used as a reference data set for comparison. Theoretical factor mix 1 (FM1) represents the original composition reported in the PURE system described by Shimizu *et al.* (2001) (*68*). It is hypothesized that the commercial factor mix closely resembles the

composition of the original PURE system, as this is the system it was based on. Overall, higher relative amounts of protein were reported in the original PURE system. It is important to note that a large difference in EF-Tu concentration would significantly impact the relative ratios of all other proteins between different conditions. Alternatively, large differences indicate further optimization is required to identify the optimal concentration of each protein.

Theoretical factor mix 2 (FM2) represents the composition of the homemade factor mix described in Lavickova *et al.* (2019), which the in-house factor mix was based on (*72*). Comparing between the two systems reveals some differences in protein abundances. For example, a lower concentration of IF1 was identified in the in-house mix, while overall similar relative abundance levels were observed. Low levels of some proteins may be limiting system performance, suggesting an increase in specific protein concentrations is needed. Overall, proteomic analysis of the factor mixes can be used as a quality control tool to track protein abundance levels between preparations and different types of systems, as well as guide troubleshooting approaches.

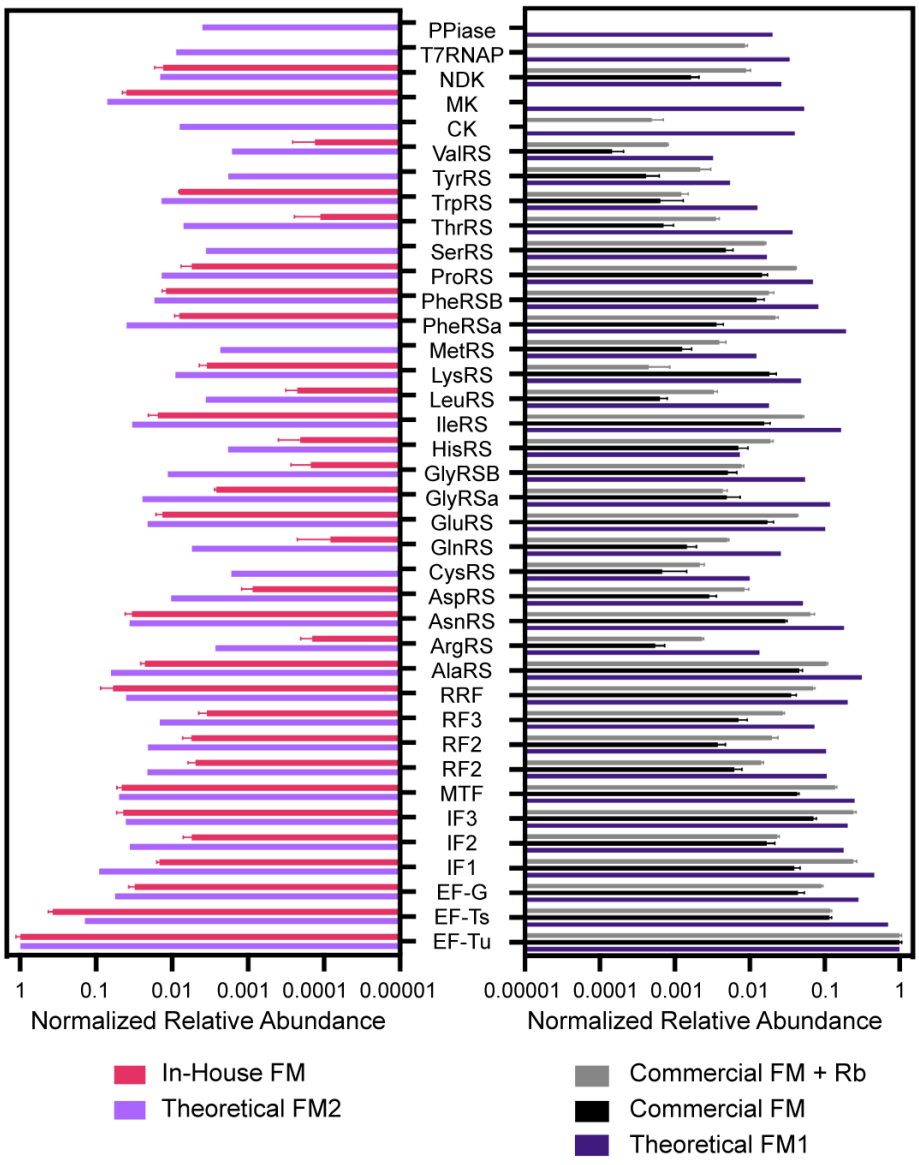


Figure 3-4: Comparing factor mix compositions reveals select proteins are present in unexpected amounts, impacting the relative ratios between components.

Relative abundance was determined by normalizing the iBAQ values for each factor to EF-Tu for the commercial (FM) with ribosomes (Rb), commercial factor mix (FM), and in-house factor mix (FM). Reported concentrations for the factor mix composition reported in Shimizu *et al.* (2001) (*68*) (theoretical FM1) and Lavickova *et al.* (2019) (*72*) (theoretical FM2) were also normalized to EF-Tu, as a comparison. Inserts show a zoomed in representation of the data.

3.4.4 IDENTIFICATION OF ADDITIONAL FACTORS AND RELEVANCE TO GENE EXPRESSION

The presence of additional factors identified in the purified *in vitro* TX-TL system is important to consider for two reasons: 1) it may be a contaminating factor that is detrimental to, or has a negative impact on, gene expression, and 2) the factor may be co-purifying due to specific interactions with TX-TL components, providing potential insights into other pathways or mechanisms that are important for efficient gene expression. The identification and classification of other proteins informs the next steps towards improving cell-free TX-TL, which may include the inhibition or supplementation of specific proteins.

First, the commercial and in-house factor mix were assessed (Figure 3-5), which are composed of proteins individually purified from *E. coli* cell lysate using nickel affinity chromatography and that include some endogenous *E. coli* proteins. This is not surprising, as the co-purification of histidine-rich proteins, or proteins with surface exposed clusters of histidine residues, is a well-known problem during immobilized metal affinity chromatography (*213*). Three common contaminants that fall into this category include: 1) cAMP-activated global transcriptional regulator (CRP) and 2) glutamine-fructose-6-phosphate aminotransferase (GlmS) identified in both factor mixes, and 3) FKBP-type peptidyl prolyl-cis trans isomerase (SlyD) identified in the in-house factor mix. Supplementation of SlyD to an *in vitro* reconstituted TX-TL reaction was previously shown to improve protein yield, demonstrating a potential beneficial effect of this additional protein (*77*).

In summary, a total of 6 other proteins were identified in the commercial factor mix (including CRP and GlmS) (Table A3-1), and a total of 11 other proteins were identified in the in-house factor mix (including CRP, GlmS, and SlyD) (Table A3-2). Upon reviewing the functional roles of these proteins, it was identified that some are involved in processes potentially irrelevant to cell-free gene expression, including cellular metabolism, purine biosynthesis, and cell wall synthesis, or require substrates and cofactors that are not present in the system. A few interesting contaminants to consider include thiole:disulfide interchange protein (DsbA) in the commercial factor mix, which aids in disulfide bond formation, and GTP cyclohydrolase I (GTP-CH-1) also

present in the in-house mix, which is known to bind GTP. DsbA highlights the need to supplement specific factors to aid in the expression of disulfide-bonded proteins in a cell-free expression system, while GTP-CH-1 may have an effect on NTP levels.

Next, the complete ribosome proteome was characterized. In summary, 227 proteins were identified in the commercial ribosomes, with 173 of these proteins classified as non-ribosomal (Table A3-3). Fewer proteins were identified for the in-house ribosomes, with a total of 143 proteins, 89 of which were non-ribosomal (Table A3-4) Comparing between the two ribosome preparations, 86 non-ribosomal proteins were common. Therefore, 87 unique non-ribosomal proteins were identified in the commercial ribosomes, and 3 non-ribosomal proteins were identified in the in-house ribosomes (Figure 3-5). For each ribosome preparation, the non-ribosomal protein components comprise <1% of the total relative abundance. Although this value is low, it is difficult to rule out the potential impact on gene expression, as it is possible for proteins of low concentrations to have an effect depending on their requirements for activity (i.e. availability of substrates or cofactors and mechanism of action).

A gene ontology (GO) analysis was performed to classify the non-ribosomal proteins and identify if specific groups of proteins were enriched. When classifying by molecular function and protein class, the majority of contaminating factors were assigned the GO terms “catalytic activity” or “binding”, and primarily identified as “metabolic interconversion enzymes” or “translation proteins” (Figure 3-5). Select proteins expected to have an impact on gene expression were identified and are discussed further (Figure 3-6).

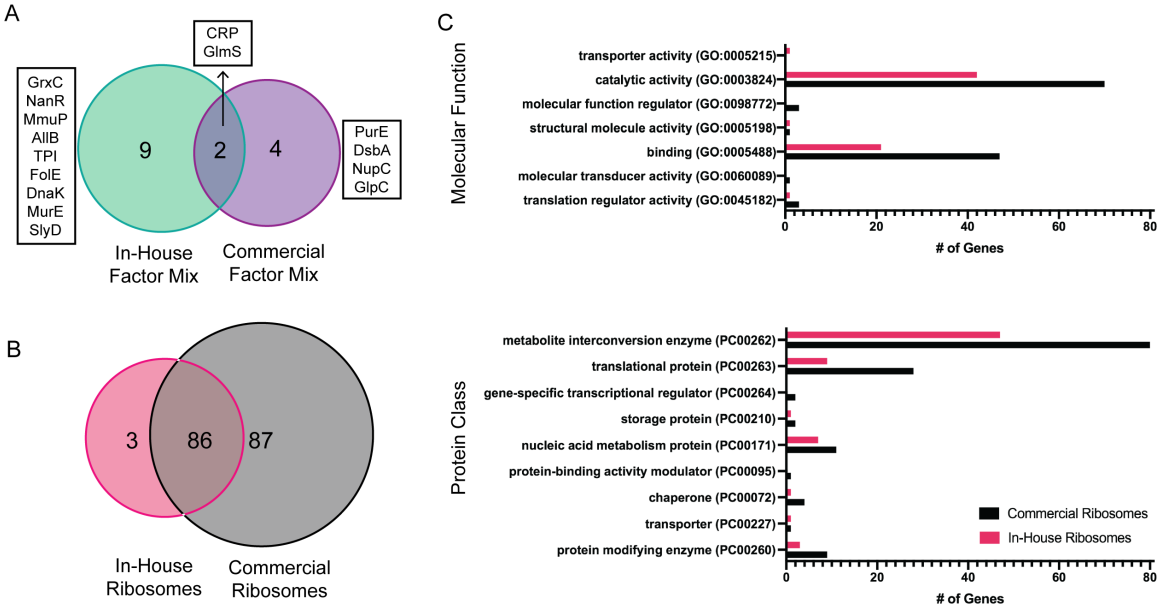


Figure 3-5: Factor mix and ribosomes contain additional factors that may have an effect on gene expression.

(A) Summary of other factors identified in the in-house and commercial factor mix and (B) ribosomes. (C) Gene ontology analysis of non-ribosomal proteins classified by molecular function and protein class.

The presence of additional translation factors is highly relevant as this will increase the final concentration of translation proteins and change the expected relative ratio between components compared to what is defined in the factor mix composition. Specifically, EF-Tu, EF-Ts, and EF-G were detected in both ribosome preparations, as well as five aaRS's. An additional ten aaRS's, IF3, RF3 and RRF were identified in the commercial preparation. It is important to consider how changes in protein abundance relates to the optimal protein concentration and what impact this has on gene expression. For example, overexpression of IF3 *in vivo* is known to decrease re-initiation efficiency (*229*), while increasing the concentration of IF1, IF2, and IF3 has been shown to inhibit translation in an *in vitro* reconstituted context (*74*). Furthermore, these values are used as inputs for ordinary differential equation modelling to study complex dynamics of translation (*157*).

An understanding of protein levels in the *in vitro* reconstituted cell-free system supports accurate computational modeling of *in vitro* gene expression.

Three unique proteins were identified in the in-house ribosome preparation, multidrug resistance protein (MdtC), ribonuclease E (RNase E), and hydroxymethylpyrimidine/phosphomethylpyrimidine (HMP) kinase. MdtC and HMP kinase are both not expected to have any significant effect. Additional factors are required for MdtC activity, while HMP kinase is involved in a multi-step enzymatic process unrelated to cell-free gene expression (*230, 231*). Alternatively, RNase E is an endoribonuclease involved in both the processing and decay of RNA and may result in a reduction in mRNA levels (*232*). Additional factors involved in mRNA degradation processes, include enolase and ribonuclease R (RNase R) identified in both ribosome preparations and exoribonuclease 2 (RNase II) identified in the commercial ribosomes. Enolase is primarily a glycolytic enzyme responsible for the dehydration of 2-phosphoglycerate to phosphoenolpyruvate. A small amount of enolase is known to bind to RNase E as a part of the RNA degradosome. Its functional role in this process is unclear (*233*), but some studies suggest enolase is involved in the degradation of specific mRNAs that encode for proteins associated with glycolytic pathways (*234*). RNase R and RNase II are both 3'-5' exoribonucleases and contribute to secondary digestion of the fragments produced from RNase E (*234*). Both degrade a wide range of substrates, but RNase R is capable of degrading highly structured RNA due to its helicase activity (*235*). Overall, the presence of RNases in a highly purified system highlights the importance of including RNase inhibitors to maintain mRNA levels.

Other RNA binding proteins identified include the RNA-binding factor Hfq and RNA-binding protein YhbY in both ribosome preparations, and the RNA chaperone ProQ in the commercial ribosomes. RNA binding proteins are of interest due the potential to inhibit or promote translation. For example, this may occur by binding to the mRNA and blocking the ribosome binding site or unwinding highly structured mRNAs and increasing accessibility of the ribosome binding site. Hfq is known to bind and unwind mRNAs and has also been reported to bind DNA (*236*). Both Hfq and ProQ are involved in modulating gene expression by acting as RNA chaperones facilitating regulatory RNA (i.e. sRNA) and mRNA interactions (*237*). YhbY is considered to be

uncharacterized, and its specific function is unknown, but one structural study suggests a role in translation due to structural similarity to the C-terminal domain of IF3, which is known to bind to the 16S rRNA (*238*). Therefore, YhbY is an interesting candidate to test for its effect on translation initiation.

Factors known to associate with the ribosome were also detected, including ribosome silencing factor (RsfS), in both preparations, and ribosome associated inhibitor A (RaiA), 30S ribosome binding factor (RbfA), ribosome hibernation promoting factor (HPF), and ribosome binding ATPase YchF, in the commercial ribosomes. RsfS and RaiA are both reported to inhibit translation. RsfS prevents the association of the 30S and 50S subunits by interacting with L14 (*239*), while RaiA has been suggested to block the A-site and P-site of the ribosome, preventing translation initiation (*240*). RbfA is implicated in 16S rRNA processing, and as indicated by its name, binds to the small ribosomal subunit (*241*). HPF binds to 70S ribosomes and, in combination with ribosome modulation factor (RMF), promotes dimerization to form 100S ribosomes. RMF is a small protein (~6.5 kDa) and may be difficult to detect by mass spectrometry. If RMF is also present, HPF and RMF may be inactivating a small portion of the ribosomes (*242*). Lastly, YchF has been reported to bind to the 70S ribosome and is hypothesized to be involved with ribosome function and/or ribosome biogenesis (*243*).

Similar to concerns of RNase contamination, proteases were also detected that could degrade the protein of interest or the translation machinery. The commercial ribosomes contained the cytosol non-specific dipeptidase (PepD), peptidase B (PepB), and both components of the ATP-dependent protease complex HslU-HslV (*244*). On the other end of the spectrum, protein chaperones were identified. The commercial ribosomes contained the chaperone protein ClpB, 10 kDa chaperonin GroES, and the chaperone protein HtpG. Both ribosome preparations contained trigger factor, the 60 kDa chaperonin GroEL, and chaperone protein DnaK. Chaperones aid in proper protein folding and may prevent protein aggregation, therefore may be beneficial to the *in vitro* reconstituted TX-TL system for the expression of functional protein. Previous studies have supplemented *in vitro* reconstituted TX-TL systems with select chaperones and observed varying results. For example, the addition of GroEL/GroES improved function and yield for the expression

of Fluc and βgal, but no improvement was observed for the expression of mCherry (*74*). Similarly, varying effects have been reported following the addition of DnaK, suggesting the effect might be protein specific (*74, 245*). Trigger factor, which is also considered a Ni^{2+}-NTA contaminant, has also been reported to improve functional yield of GFP (*77*). Since some chaperones are already present in the system, again it is important to consider what effect this has on the optimal and final protein concentration. From our analysis, it may be of interest to test the addition of other chaperones, that have yet to be supplemented that co-purify with ribosomes, including ClpB and HtpG.

A top common hit for both ribosome preparations was SsrA-binding protein (SmpB). SmpB is an important component of the primary ribosome rescue mechanism in *E. coli*. Ribosome stalling caused by non-stop (truncated) mRNAs require a sophisticated mechanism to alleviate the stalled ribosome complex and maintain translation capacity of the system. Referred to as *trans*-translation, SmpB in complex with transfer messenger RNA (tmRNA) alleviates the stalled ribosome complex, tags the truncated protein for degradation, and releases the mRNA to be degraded by exoribonucleases (RNase R) (*246*). The presence of SmpB in the purified ribosomes suggests that a portion of the ribosomes are stalled *in vivo* prior to isolation. More interestingly, since ribosome stalling and the production of truncated proteins has been reported as a main issue with *in vitro* gene expression, the presence of SmpB suggests that a mechanism to alleviate ribosome stalling needs to be included in the *in vitro* system to maintain translation fidelity. *Trans*-translation requires the concerted action of proteases and RNases to degrade the truncated protein and mRNA, ultimately complicating how to implement these quality control mechanisms in an *in vitro* context without running into the risk of degrading other functionally relevant proteins and mRNAs. Two alternative ribosome rescue pathways have been tested in an *in vitro* context, involving alternative ribosome rescue factor A (ArfA) and alternative ribosome rescue factor B (ArfB). The addition of ArfA resulted in an increase in Fluc fluorescence (with no stop codon), while no effect was observed following the addition of ArfB (*76*). Overall, the incorporation of ribosome rescue mechanisms in a cell-free expression system presents a challenging engineering problem to address and a rescue mechanism involving SmpB has not been tested yet.

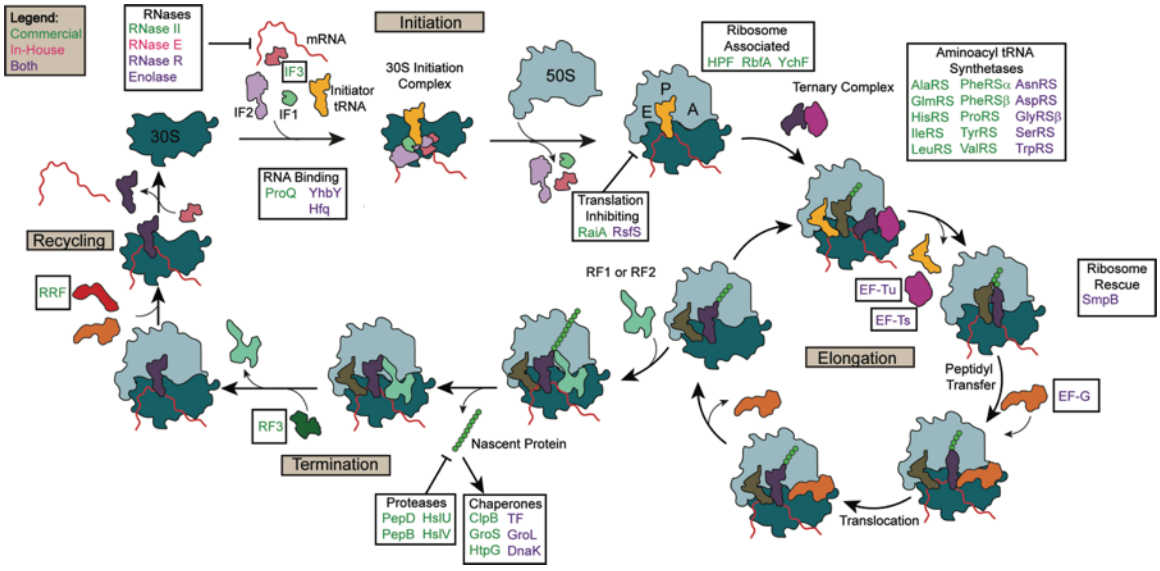


Figure 3-6: Additional factors identified from the proteomic analysis are involved in gene expression.

Proteins identified from the proteomic analysis of ribosomes are mapped to the process of translation. Green = identified in the commercial system, Pink = identified in the in-house system, Purple = identified in both the commercial and in-house systems. Adapted from (*247*) and (*248*).

3.4.5 RECOMMENDATIONS FOR IMPROVED GENE EXPRESSION *IN VITRO*

A complete proteomics analysis of a minimally reconstituted *in vitro* TX-TL system provides important insights about ribosomal protein stoichiometry and the relative abundance of key factors required for TX-TL. Furthermore, this approach delivers a quality control tool to monitor system composition between batches, informs the optimization of protein concentrations, and identifies contaminating factors that may be detrimental to gene expression. For example, it is important to be aware of factors that degrade RNA and protein, or NTP-dependent enzymes that alter NTP levels depleting the energy currency of the system. Additional factors hypothesized to be beneficial to gene expression are also identified and recommended to be supplemented into the *in vitro* reaction. Promising candidates include, the RNA binding protein YhbY, RNA chaperones ProQ and Hfq, protein chaperones HtpG and ClpB, and factors important for disulfide bond formation. Lastly,

an engineered approach to alleviate ribosome stalling and provide a mechanism for ribosome rescue (e.g., involving SmpB) is an important future direction that may resolve issues of poor protein yield and the expression of truncated products.

Comparisons to S30 lysate proteomic data lead to the identification of other factors that are missing and might be important for *in vitro* gene expression. For example, three additional elongation factors involved in translational fidelity have been reported: EF-4, elongation factor P (EF-P), and elongation factor P-like protein (YeiP) (*59, 61*). EF-4 has been suggested, as one of its proposed roles, to catalyze back translocation of a defective post translocation complex (*249*), while EF-P alleviates ribosome stalling on consecutive polyproline residues (*250*). YeiP may also be implicated in ribosome rescue as it is hypothesized to be a paralogue of EF-P, but little is known about its function. EF-4 and EF-P have both been supplemented to an *in vitro* reconstituted TX-TL system and resulted in improved protein synthesis (*69, 71, 74, 76*), while YeiP has yet to be tested and might be beneficial to gene expression. An additional factor not identified in our proteomic analysis, but referred to as important component in previous studies includes the heat shock protein 15 (HslR) as a potential ribosome recycling factor (*59*). HslR helps to recover free 50S subunits that may still be associated with a nascent peptide chain, providing another approach to assist in ribosome recycling.

3.5 CONCLUSION

The proteomic examination of an *in vitro* reconstituted cell-free TX-TL system reveals useful insights into the system composition. The resulting analysis pipeline provides utility as a promising approach to reduce batch-to-batch variation and for the identification of factors important to the performance of the *in vitro* biomolecular machinery. For example, the inclusion of specific RNase inhibitors or protease inhibitors is important to reduce mRNA and protein degradation. An accurate understanding of translation factor relative abundance also guides the optimization of protein concentration, which is affected by the co-purification of select factors with ribosomes. From this analysis, select candidates have been suggested for further testing. Overall a proteomic analysis

of the components of a cell-free system provides an additional tool to supplement previous studies aimed at improving *in vitro* reconstituted gene expression.

CHAPTER 4 - RIBOSOMAL PROTEIN S1 IMPROVES THE PROTEIN YIELD OF AN *IN VITRO* RECONSTITUTED CELL-FREE SYSTEM

4.1 PREFACE

Chapter 4 was written and published as a technical note in the journal *ACS Synthetic Biology*. The manuscript has been reformatted for this thesis.

T. Sheahan and H.-J. Wieden, Ribosomal protein S1 improves the protein yield of an *in vitro* reconstituted cell-free system. *ACS Synthetic Biology* **11**, 1004-1008 (2022). doi: 10.1021/acssynbio.1c00514

Contributions of Authors: Dr. Hans-Joachim Wieden and I conceived the study and together wrote the manuscript. I performed all experiments and data analysis

Overall, this chapter addresses a current shortcoming of cell-free systems and describes an improvement to the protein synthesis capacity based on my observation that ribosomal protein S1 is significantly under-represented in the in-house purified ribosomes, suggesting it as a potential component to increase ribosome performance *in vitro.*

4.2 INTRODUCTION

The ability to efficiently perform transcription and translation (TX-TL) outside of a living organism, unimpeded by inherent cellular processes, is a powerful tool for a wide range of emerging biotechnology applications. Referred to as cell-free coupled TX-TL systems, these methods offer a flexible design platform and a reduced biocontamination risk, leading to applications in sustainable biomanufacturing, healthcare, and agriculture (*46*). Cell-free systems are composed either of crude cell extracts (*35*) or highly purified *in vitro* reconstituted components (*68*). Highly purified systems, such as the commercially available PURExpress® derived from *Escherichia coli*, have the added benefit compared to cell extracts of a minimal and defined composition, excluding nucleases and

proteases, and consist of ribosomes, TX-TL factors, NTPs, tRNA, amino acids, and other cofactors. Purified *in vitro* reconstituted systems can be produced with tailored compositions and components can be easily added or removed (including the ability to swap in components derived from different organisms). In addition to providing a simplified approach for elucidating TX-TL mechanisms, cell-free systems are highly amenable for advances in genetic code expansion allowing for novel protein functions to be unlocked (*214, 251*), and for developments in artificial self-regeneration building toward the construction of a minimal cell (*217, 252*).

A major limitation of *in vitro* reconstituted TX-TL systems, such as PURExpress®, which restricts their full utilization for biotechnology development and commercial applications, is a low protein yield, ranging from 10 – 200 µg/mL (see New England Biolabs PURExpress® manual (*215*)). This is much lower than for *E. coli* cell extracts, which yield ~1000 µg/mL of protein (*216*), suggesting further optimization is required to improve the overall performance of *in vitro* reconstituted TX-TL systems. Furthermore, highly pure cell-free systems often have difficulty translating structured RNA (*215*), limiting the design space with respect to the types of mRNA that can be translated.

Previous studies have identified ribosomes and ribosome-associated processes as the main culprit for poor system performance. This includes 1) poor ribosome processivity (*76, 82*), 2) inefficient ribosome recycling (*76*), and 3) inefficient translation initiation (*81*), leading to truncated products and/or low protein yields. In the latter case, Doerr *et al.* (2019) reported translation initiation to be the rate limiting step and identified that only a small portion of ribosomes were actively translating (*81*), which may be further impacted by mRNA secondary structure inhibiting access to the RBS. In general, we experience similar limitations in ribosome performance when analyzing band intensities of the translated product, DHFR, observing ~50% intensity relative to EF-Tu present in the reaction (Figure A4-1). EF-Tu is a core component of the translation machinery delivering aminoacyl-tRNA to the ribosome for amino acid incorporation into the growing polypeptide chain. Furthermore, estimating from the band intensity the amount of DHFR produced reveals equimolar amounts relative to ribosomes present in the reaction, suggesting that either only

a small fraction of ribosomes are initiating translation or that only few rounds of protein synthesis take place per ribosome.

To address these well-known issues, fed-batch or continuous exchange reactions have been employed to remove toxic by-products and replenish important factors required for efficient translation (*76, 218*). Improved yields have been obtained through the optimization of component concentrations, including EFs, IFs, RFs, RRF, and Mg^{2+} (*74, 218*). Lastly, supplementing with factors that target specific processes, such as EF-P to alleviate ribosome stalling (*76*) and EF-4 to aid in back translocation following a defective translocation reaction (*76*), has been beneficial to system performance. Other examples include the addition of chaperones, such as GroEL/ES, to aid in protein folding, and molecular crowding agents, such as BSA, to better mimic the *in vivo* environment (*74*). Here, we provide a new approach aimed at targeting translation initiation and show the importance of ribosome composition for efficient translation, adding to the toolbox of optimizing *in vitro* reconstituted TX-TL systems.

Ribosomal protein S1 plays an important role in translation initiation by recruiting mRNA to the 30S subunit (*253*). S1 exhibits RNA helicase activity, facilitating the unfolding and correct positioning of mRNA on the ribosome (*254*) and is essential for translating mRNAs with weak and/or structured RBSs (*255*). Recent work by Romilly *et al.* (2020) demonstrated the requirement for S1 in standby-mediated translation and the unwinding of an RNA pseudoknot upstream of a coding sequence to allow optimal 30S binding (*256*). S1 has also been reported to loosely bind to the 30S subunit, and therefore, purified 70S ribosomes often have a lower S1 to 70S ratio compared to those *in vivo (228)*. In line with these reports, less than stoichiometric amounts of S1 have been observed following SDS-PAGE analysis of purified ribosomes and in the comparison of band intensities of S1 to those of core ribosomal protein L2 (Figure A4-2). Due to the functional role of S1 in translation initiation, as well as its ability to unfold structured RNA, we hypothesized that increasing its availability in a cell-free reaction can improve system performance.

4.3 METHODS

4.3.1 PLASMIDS AND STRAINS

Genes encoding mRFP (BBa_E1010) and eCFP (BBa_E0020) were obtained from the registry of standard biological parts (http://partsregistry.org) and sub-cloned into pSB1C3. The EYFP construct was synthesized as a gBlock gene fragment from Integrated DNA Technologies (IDT) and sub-cloned into pJET (CloneJET PCR Cloning Kit, Thermo Fisher Scientific). The sfGFP plasmids were obtained from Roberts and Wieden (*under review*). All constructs were sequence confirmed by Genewiz. Plasmid DNA was amplified in *E. coli* DH5α and purified using the EZ-10 Spin Column Plasmid DNA Miniprep Kit (BioBasic). Plasmid concentration and purity was determined by measuring the absorbance at 260 nm and 280 nm using spectrophotometry (BioDrop DUO UV/VIS Spectrophotometer, Biochrom, Ltd.). The respective DNA sequences are summarized in Table A4-1.

4.3.2 RIBOSOMAL PROTEIN S1 EXPRESSION AND PURIFICATION

The purification protocol was adapted from Duval *et al.* 2013 (*255*). In detail, N-terminal hexahis-tagged ribosomal protein S1 was purified from the rpsA clone of the *E. coli* ASKA library(*257*). *E. coli* cells were grown in LB media supplemented with 35 µg/mL chloramphenicol. S1 expression was induced at an $OD_{600\ nm}$ of ~0.6 with IPTG (#IB0168, BioBasic) to a final concentration of 1mM. Three hours after induction, cells were harvested by centrifugation at 5000 xg for 20 min, flash frozen with liquid nitrogen, and stored at -80°C. To purify S1, the cell pellet was thawed on ice and resuspended in 5 mL/g (cells) of binding buffer (50 mM Tris-HCl pH 8.0 at 4°C (#BP152, Fisher BioReagents), 40 mM NH_4Cl (#A15000, Alfa Aesar), 7 mM $MgCl_2$ (#M8266, Sigma Aldrich), 7 mM β-mercaptoethanol (#444203, Millipore Sigma), 300 mM KCl (#P217, Fisher BioReagents), 10 mM imidazole (#IB0277, BioBasic), 15% glycerol (#BP229, Fisher BioReagents), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF, #PB0425, BioBasic)). To lyse the cells, 1 mg/mL of lysozyme (LDB0308, BioBasic) was slowly added at 4°C and incubated for 30 min, followed by the addition of 12.5 mg/g (cells) of sodium deoxycholate (#89904, Thermo Scientific) at 4°C and incubated for 60 min. The cell lysate was then centrifuged at 30 000 xg for 30 min at 4°C. The resulting supernatant was incubated with 100 µg/mL of RNase A (#RB0473, BioBasic) for 2.5-hours

at 4°C. To dissociate S1 from ribosomes, NH_4Cl was added to a final concentration of 1M, followed by centrifugation at 45 000 xg for 2-hours. The resulting S1-containing supernatant was applied to 5 mL of Ni^{2+} Sepharose resin (GE Healthcare), which was equilibrated with binding buffer, and incubated for 1-hour at 4°C. To remove the unbound proteins, the resin was collected by centrifugation at 500 xg for 2 min. To remove any low-affinity bound protein, the resin was incubated with 40 mL of wash buffer (binding buffer with 20 mM imidazole), incubated at 4°C while shaking, followed by centrifugation at 500 xg for 2 min and subsequent decanting of the supernatant. The wash step was repeated 3 additional times. To elute the protein, 4 mL of elution buffer (binding buffer with 250 mM imidazole) was added to the resin, mixed, incubated at 4°C for 5 min and centrifuged at 500 xg for 2 min. Elution was repeated for a total 10 times, all elution fractions were subsequently pooled, and concentrated to 5mL at 45 µM using a spin column (Vivaspin 30, GE Healthcare). To remove any RNA co-purifying with S1, the concentrated protein was applied (ÄKTAprime Plus, GE Healthcare) to a Mono-Q GL column (GE Healthcare, 17-5166-01) equilibrated with 20 mM Tris-HCl pH 7.5 at 4°C, 40 M NH_4Cl, 60 mM KCl, 1 mM DTT, and 10% glycerol. The protein was eluted using a gradient (40 mM – 1 M NH_4Cl, 0.5 mL/min). Fractions containing S1 were identified using SDS PAGE, pooled, and subsequently rebuffered (20 mM Tris-HCl pH 7.5 at 4°C, 40 mM NH4Cl, 60 mM KCl, and 1 mM DTT) and concentrated using a Vivaspin concentrator (Vivaspin 30, GE Healthcare, MWC 30 kDa). The purified protein (typically >90% using Coomassie blue staining) was aliquoted, flash frozen with liquid nitrogen, and stored at -80°C. The protein concentration (240 µM) was determined spectroscopically using the molar extinction coefficient of ε_{280} = 47 656 $M^{-1}cm^{-1}$ (determined using the ProtParam tool (https://web.expasy.org/protparam/) and the amino acid sequence) at 280 nm (BioDrop DUO UV/VIS Spectrophotometer, Biochrom, Ltd.).

4.3.3 *IN VITRO* TX-TL REACTIONS

In vitro TX-TL reactions were performed using the PURExpress® kit from New England Biolabs (#E6800). Reactions were performed according to manufacture instructions with the following modifications. In brief, 10 µL of Solution A, 7.5 µL of Solution B, 250 ng of plasmid DNA, and 0.5 µL of RNase Inhibitor (RiboLock, #E00381, Thermo Fisher) were combined with milliQ H_2O

resulting in a final reaction volume of 25 µL. Test reactions included ribosomal protein S1 (2.4 µM, 4.8 µM, 7.2 µM, 9.6 µM, or 12 µM) in excess relative to ribosome concentration (2.4 µM), in a final reaction volume of 25 µL. Individual reactions were incubated overnight (16-hours) at 37°C and placed on ice after to stop the reaction. In each set of experiments, a negative control was included, where milliQ H_2O was added instead of the respective template DNA. All reactions were performed in triplicate.

4.3.4 FLUORESCENCE MEASUREMENTS

The produced fluorescent proteins were excited at the wavelength optimal for the respective fluorescent reporter protein and the corresponding emission spectra were recorded (QuantaMaster Fluorimeter (Photon Technology International (Canada) Inc.). 25 µL PURExpress® reactions were diluted to a final volume of 150 µL with $TAKM_7$ buffer (50 mM Tris-HCl pH 7.6 at 4ºC, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$) and placed in a quartz cuvette (Starna Cells Inc.). EYFP was excited at 513 nm (± 1.5 nm) and emission scanned between 525-625 nm (± 2.5 nm, λ_{max} = 527 nm). sfGFP was excited at 488 nm (± 1.5 nm) and emission scanned between 503-603 nm (± 2.5 nm, λ_{max} = 508 nm). mRFP was excited at 584 nm (± 1.5 nm) and emission scanned between 599-699 nm (± 2.5 nm, λ_{max} = 607 nm). eCFP was excited at 439 nm (± 1.5 nm) and emission scanned between 454-554 nm (± 2.5 nm, λ_{max} = 470 nm). Prior to analysis, the negative control was subtracted from each emission spectra.

4.4 RESULTS AND DISCUSSION

To assess the impact of S1 on the protein yield of a coupled TX-TL system, ribosomal protein S1 was added at a 1 to 1 ratio relative to the ribosome concentration (2.4 µM of S1 added to a reaction with 2.4 µM of ribosomes), along with template DNA encoding a fluorescent protein. Protein expression was subsequently compared using the autofluorescence of different fluorescent reporter proteins. With EYFP, a 20% increase in peak fluorescence was observed following addition of S1 (Figure 4-1A). Further increasing the concentration of S1 up to 12 µM increased EYFP yields by 50% while the addition of the crowding agent, BSA, had a minimal effect on EYFP fluorescence

(Figure A4-3). Overall, consistent with our hypothesis, supplementing the TX-TL reaction with S1 improved protein yields as indicated by an increase in observed fluorescence.

Improved system performance was also observed for the expression of other fluorescent proteins, demonstrating the positive effect of S1 in the context of different coding sequences. The addition of 2.4 μM S1 increased sfGFP fluorescence by 60% and mRFP fluorescence by 50% (Figure 4-1B). Interestingly, no effect was observed for the expression of eCFP. To determine if the latter was concentration dependent, different amounts of S1 were tested but in all cases no significant increase in fluorescence was observed (data not shown). The genes for mRFP and eCFP, respectively, are located in the multicloning site of pSB1C3 downstream of the same promoter and RBS. It is therefore unclear why the addition of S1 does not have a positive effect on eCFP expression, suggesting that other factors are also influencing translation efficiency, such as codon usage, and that eCFP expression is not limited by S1-facilitated initiation.

For all four reporter proteins, expression was controlled by a strong RBS (BBa_B0034). To test the effect of S1 on translation in a different 5'UTR context, the strong RBS was replaced with a medium (BBa_B0033) and weak (BBa_B0032) RBS upstream of the sfGFP coding sequence. In both cases, an increase in fluorescence was observed corresponding to an 80% and 110% increase in protein yield for the medium and weak RBS, respectively (Figure 4-1C and 4-1D). S1 had a greater effect on protein yield as the RBS strength decreased, consistent with S1's role in the translation of mRNAs with weak RBSs (*255*). To investigate if S1 can also improve the translation of a highly structured RNA, sfGFP was placed under the control of the internal ribosome entry site (IRES) from *Plautia stali* intestine virus (PSIV), which contains three pseudoknot structures (Figure A4-4) (*258*). With the of S1, sfGFP fluorescence increased by 60%, demonstrating improved translation of a structured RNA upstream of the coding sequence (Figure 4-1C and 4-1D).

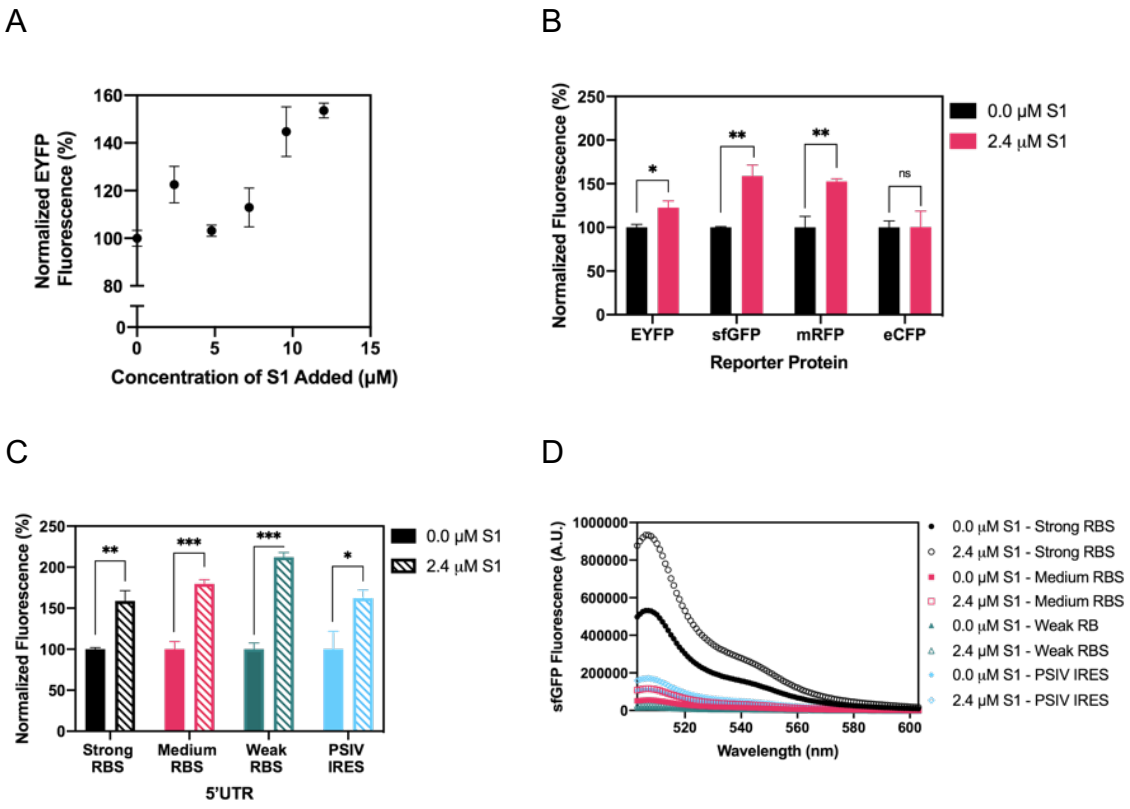


Figure 4-1: Ribosomal protein S1 improves the protein yield of an *in vitro* reconstituted TX-TL system.

Effects of (A) increasing concentration of ribosomal protein S1 (2.4 μM, 4.8 μM, 7.2 μM, 9.6 μM, or 12 μM) on EYFP fluorescence, (B) S1 added in excess (2.4 μM) relative to ribosome concentration (2.4 μM) on EYFP, sfGFP, mRFP, and eCFP fluorescence, and (C) excess S1 (2.4 μM) on sfGFP expression with different 5'UTRs. D) Representative emission spectra of sfGFP fluorescence with or without additional S1 (2.4 μM), under the control of a strong RBS, medium RBS, weak RBS or PSIV IRES. EYFP excitation = 513 nm, λ_{max} = 527 nm; sfGFP excitation = 488 nm, λ_{max} = 508 nm; mRFP excitation = 584 nm, λ_{max} = 607 nm; eCFP excitation = 439 nm, λ_{max} = 470 nm. Note that in (A), (B), and (C) fluorescence measurements are normalized to reactions without additional S1 at the peak emission wavelength. Statistical significance was determined using a multiple t-test (Holm-Sidak method), with α = 0.001 (*** p≤0.001) α = 0.01 (** p≤0.01) or α = 0.05 (*p ≤0.05), (n=3) (GraphPad Prism software).

The increase in fluorescence following addition of S1 is comparable to results reported in previous studies when supplementing *in vitro* reconstituted cell-free systems with translation factors. For example, supplementing with EF-4 improved yields by 30% to 100% depending on the reporter protein used (Fluc, β-gal, or mCherry) (*74*), an overall improvement similar to the change in expression observed here with additional S1. Previous studies have also shown a combinatorial effect when supplementing the reaction with multiple factors resulting in further increases in protein expression (*74, 76*). Therefore, coupling the addition of S1 with previously reported factors that address different limitations of the reconstituted cell-free TX-TL system will likely further improve protein yields beyond the levels reported here. Our results suggest that S1 aids in improving translation initiation, consistent with its cellular function and a lack of S1 occupancy in purified ribosomes.

In addition to improving cell-free protein expression, which is an important parameter for synthetic biology applications, these results also provide a step toward understanding additional roles of S1. It is well understood that S1 is essential for translation initiation; however, S1 has been suggested to also play a role in processes such as translation elongation (*259*) and transcriptional cycling (*260*). As such, the observed S1 concentration-dependent change in EYFP fluorescence (Figure 1A) suggests two functional mechanisms. A potential explanation includes improving the fraction of initiating ribosomes by binding S1 to S1-vacant ribosomes, resulting in an initial increase in EYFP fluorescence. As S1 concentration increases, free S1 can also bind and stabilize the structure of the mRNA, further improving translation efficiency, ultimately resulting in an additional increase in EYFP fluorescence at higher S1 levels. Regarding the detailed function of S1, further mechanistic analyses are required to determine if the observed effect of increasing S1 concentration is the result of improved ribosome recruitment or if there are additional mechanisms at play. For example, RNA folding may be impacted by the high processivity of T7 phage RNA polymerase. Therefore, mRNA binding by free S1 may be important for enhanced translation through RNA stabilization.

4.5 CONCLUSIONS

Improving the performance of cell-free systems will allow for the rapid, easy, and sustainable production of important materials, chemicals, and compounds, and for the engineering of biological and life-similar systems. To enable such applications, a continued interest in refining cell-free expression systems is vital. Overall, we have identified an additional venue to increase protein yield by supplementing a reconstituted *in vitro* translation system, such as the commercial PURExpress® reaction, with recombinant ribosomal protein S1. Yield improvement was demonstrated for different reporter proteins and in different 5'UTR contexts. Furthermore, S1 aids in the translation of structured mRNA, addressing another limitation of the PURExpress® system. These results build toward a highly functional reconstituted cell-free expression system by targeting translation initiation and highlighting the critical role that the ribosome composition plays for improving the utility of cell-free expression systems in biotechnology development and next generation cell-free synthetic biology.

CHAPTER 5 - EMERGING REGULATORY CHALLENGES OF NEXT-GENERATION SYNTHETIC BIOLOGY

5.1 PREFACE

Chapter 5 was published in *Biochemistry and Cell Biology* as a part of a collection entitled *Dual Use of Research in the Life Sciences*.

T. Sheahan and H.-J. Wieden. Emerging Regulatory challenges of next-generation synthetic biology. *Biochemistry and Cell Biology* **99**, 766-771 (2021). doi: 10.1139/bcb-2021-0340

Contributions of Authors: Dr. Hans-Joachim Wieden and I wrote the manuscript. I conducted the literature review and prepared the figure.

While working with cell-free systems and observing the advent of more commercial uses, the real-world applications and societal benefits of cell-free biotechnology was apparent. However, policy and regulation associated with cell-free biotechnology was unclear and there is a potential for misuse. Therefore, this manuscript aims to initiate a policy discussion and provide a stepping stone for the development of guidelines to assess next-generation synbio and inform policy development.

5.2 INTRODUCTION

Synbio has emerged as a scientific field with tremendous transformative potential, providing critical tools for achieving the sustainable development goals identified by the United Nations (UN) (*261*). Through the rational engineering of living systems, synbio-inspired biotechnology has the potential to deliver alternative solutions to combat global food shortages, climate change, energy resource depletion, and therapeutic limitations (*2*). However, current approaches depending on re-purposing and re-programming existing living systems (top-down) impose limitations that need to be overcome to harness the full potential of synbio. The bottom-up

design of life-similar systems and devices that function outside the constraints of living cells (i.e. cell-free synbio) side-step these limitations and are gaining traction in both academia and industry.

Life-similar systems provide new opportunities, but also present novel risks with respect to biosafety and biosecurity. Given its global impact, it is surprising that within current regulatory conversations the emerging risks and sharing benefits associated with cell-free and life-similar biotechnology are not discussed. Among policymakers and scientists there is a debate over what is defined as “novel biotechnology,” leading to confusion over the regulation of next-generation synbio and uncertainty regarding what policies and regulations apply to cell-free systems (*262*). This potential for omission is of concern because of the speed at which disruptive technologies are developed and adopted, and the scope of their impact. Here, we provide a forward-looking assessment of the biosafety and biosecurity implications of cell-free and life-similar systems and promote a framework for global evidence-based regulatory conversations. Overall, we aim to support the continued translation of cell-free technology into products and applications, while also safeguarding public trust.

5.2.1 EMERGING ROLE OF CELL-FREE SYSTEMS FOR SYNBIO

The current, primarily top-down, approach to engineering biological systems has resulted in a variety of commercial successes ranging from artemisinic acid production in yeast for the treatment of malaria (*263*) to precise genome engineering tools such as CRISPR (*264*). However, the full potential of synbio has not yet been realized as integration with industrial infrastructure and medical practices has been challenging (*180, 265*), impacting its disruptive potential to provide novel approaches to produce biofuels, biomaterials, and therapeutics. This is in part due to the controversy surrounding GMOs, their possible impact on the environment, and corresponding containment and regulation concerns. Further compounding is the lack of regulatory security regarding the protection of intellectual property and limitations faced in the straight-forward designability of living systems counteracting their evident benefits (Figure 1).

As an alternative, cell-free systems provide life-similar functionality outside the constraints of living cells and are commercially available or prepared in-house, as cell extracts or purified

reconstituted systems. These systems, which include the necessary biomolecular machinery to produce RNA and protein, result in a reduced bio-contamination risk, as cell-free components are currently unable to replicate, propagate or evolve. Compared to cell-based approaches, cell-free systems are not limited by competing cellular processes, such as metabolism, and no cloning selection method is required. Because they are not restricted by experimental constraints, such as transformation efficiency, they enable rapid DBTL cycles. Their ease of use, reduced complexity, ability to incorporate unnatural amino acids and tolerance of toxic compounds make cell-free systems a powerful tool for synthetic biology and biotechnology development.

Consequently, cell-free systems have been successfully used to produce important biomolecules, including therapeutics, such as the cytotoxic protein onconase (*197*) and medically applicable cannabinoids (*198*) and metabolites, including the industrially relevant monoterpenes (*182*), demonstrating their commercial relevance as bio-factories. Outside their use as a platform technology, these systems enable the development of bio-devices with applications in diagnostics, such as the paper-based detection of Zika virus (*29*), bioremediation, and biosensing. Overall, cell-free systems provide a promising avenue for developing solutions to the grand challenges synbio aims to address. Their potential is especially evident when considering the negative public perceptions of GMOs. For example, cell-free approaches to bioremediation circumvent the need to release a genetically modified organism into the environment, providing a “safer” alternative, as well as aligning with well-established customer and production models.

With continued advancement, the application of cell-free systems is becoming mainstream and is not limited to classical laboratory settings. Ease of use and inherent safety allow cell-free synbio to be more accessible to the public and citizen scientists in comparison to conventional biological engineering of living systems, especially with growing trends in do-it-yourself (DIY) biology and the establishment of community labs. For example, BioBits™, an educational kit developed using cell-free systems brings synbio to the classroom, lowering the barrier for schools to use these technologies and moving towards more mainstream uses of synbio (*202, 203*).

In recent years, cell-free start-up companies have emerged, such as Arbor Biosciences (*266*) and Sutro Biopharma (*267*), which use cell-free approaches for protein engineering. Recently,

SwiftScale Biologics has pivoted the use of cell-free platform technology to combat the COVID-19 pandemic by rapidly producing antibodies (*30*). The utility of cell-free systems in times of crisis was also demonstrated by the Broad Institute during a pressure test conducted by the U.S. Defense Advanced Research Projects Agency (DARPA). When infrastructure and current production methods are compromised and cell-based approaches are not feasible due to toxicity issues, cell-free systems can be engineered to rapidly produce important biomolecules (*184*). Overall, this demonstrates use for on-demand fabrication of necessary biomolecules and medicines.

Most recently, bottom-up approaches to synbio have received considerable attention, providing an alternative route to addressing the challenges faced by engineering living systems (and derived cell-free approaches) to unlock the full potential of synbio (*268*). By using a reverse engineering approach, biological processes are refactored and reconstructed from scratch (i.e. refactoring life). Specific cellular functions are separated from the complexity of living organisms, allowing for the rational design of life-similar synthetic biological systems. Advancements in bottom-up synbio towards the ultimate goal of creating synthetic life (see the Build-a-Cell initiative (*269*)) have consequently opened the door to new biotechnology created outside of the confines of living cells and the engineering limitations imposed by the design strategies applied by living systems (e.g., genetic code and codon usage, biophysical properties, etc.). Life-similar systems build on experience made with conventional cell-free systems; they are not affected by the inherent complexities of cellular processes that have evolved to enable fitness of the cell under a wide variety of stresses and conditions, providing engineers with increased control and greater freedom of design. This is especially true in the case of purified reconstituted systems, which consist of a reduced number of specified components required to execute select life-similar functions outside of a cell.

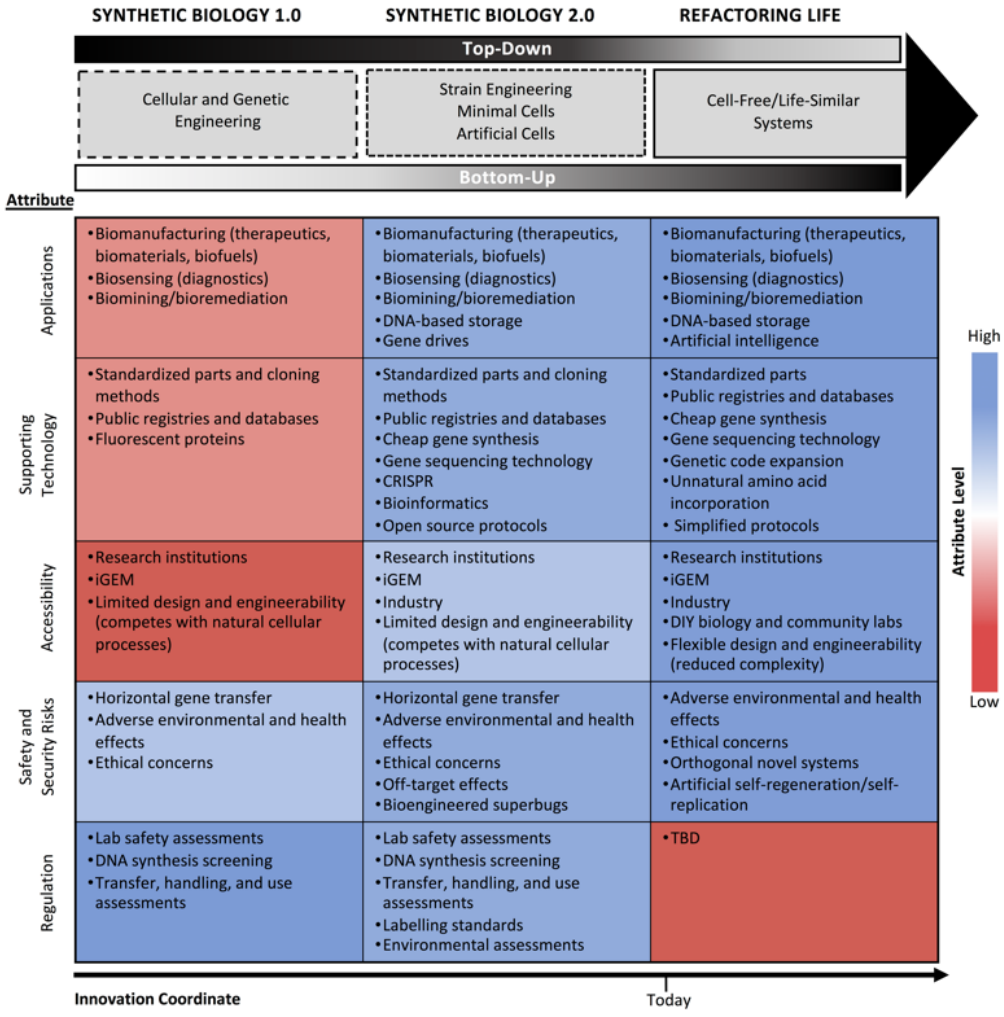


Figure 5-1: Progress of synthetic biology advancement.

At each stage of synthetic biology, the corresponding level of applications, supporting technology, accessibility, safety and security risks, and regulation is provided as a heat map. Synthetic Biology 1.0 refers to the early stages of bioengineering utilizing a primarily top-down approach, including cellular and genetic engineering; Synthetic Biology 2.0 refers to advancements that utilize both top-down and bottom-up synbio approaches, such as strain engineering, minimal cells, and artificial cells; Refactoring Life refers to the next phase of synbio, including the development of cell-free and life-similar systems through a primarily bottom-up approach. This phase is hallmarked by an increased potential of orthogonality, as well as the potential to escape from traditional monitoring and regulations applied to living systems.

5.3 BIOSECURITY AND BIOSAFETY – THE POTENTIAL FOR MISUSE

As cell-free technology evolves and becomes more commonly accessible, it is important to acknowledge the dual-use potential of these systems and their latent ability to cause harm, intentionally (i.e. biosecurity risks) or accidentally (i.e. biosafety threats), as is the case with any other human technology. Along with this, it is necessary to implement proper policies and practices, and to develop new approaches to prevent and mitigate their misuse.

The emerging biosecurity risks (e.g., bioterror) associated with synbio technology developments was highlighted in a report by the National Academies of Science, Engineering, and Medicine for the U.S. Department of Defense (*270*). The properties of bottom-up cell-free systems that make them a promising platform technology for advanced bioengineering also bear dual-use potential that can be exploited by malicious parties, including non-state actors. The current economic impact of COVID-19 demonstrates that such an act of bioterrorism does not have to cause a large number of fatalities to significantly impact society. Streamlined open-source approaches and protocols to purify cell-free systems (e.g., one-pot purification methods (*72*)) also simplify how they can be obtained and distributed, demonstrating a decreasing technology barrier, which is a hallmark of disruptive innovation potential. Furthermore, the genetic information required to program cell-free systems is easily accessible owing to the low cost of DNA synthesis. With continued commercialization and increased access, it is reasonable to predict at-home use of cell-free systems in the future. How these systems will be engineered (i.e. with increased stability), deployed, and interact with the environment may have important implications for biodiversity, requiring a full assessment of the emerging risks.

The ability to incorporate unnatural amino acids and/or re-design the genetic code creates the potential to construct new-to-nature and undetectable biomolecular devices. Previous work has already achieved expansion of the genetic code from a four to eight letter alphabet, and progress has been made towards reassigning codons to encode for non-standard amino acids (*168, 271*). Executing these novel codes in a cell-free system will evade current regulatory and screening regimes used to identify potential threats, impacting current global biosecurity measures.

Adding another layer of complexity, incorporating artificial self-replication and self-regeneration into these reduced systems to create fully synthetic bio-machines or assemblies addresses a current shortcoming of cell-free systems. In 2019, the construction of a photosynthetic artificial cell capable of regenerating energy that was used to produce its own membrane proteins was reported, providing a step closer to creating a completely independent artificial system (*138*). Progress in this area will be driven by commercial interests as these systems can utilize a proprietary genetic code (such as the eight-letter alphabet) to protect the intellectual property of the design and/or the unauthorized manipulation of these systems. Concerns of creating a deadly bioengineered “organism” that will impact humans on a population scale is now amplified by the ability to create orthogonal synthetic systems that could impact all forms of life and escape classical detection and mitigation strategies.

5.4 INNOVATION VERSUS REGULATION

Uncertainty with regarding the regulation of cell-free synbio exists, especially regarding orthogonal synthetic life-similar systems, impacting both the application and full utilization of the technology, and the social licenses to do so. Reflecting on the current public opinion of GMOs reveals a disconnect between what scientists consider safe and what the public views as dangerous. Proactive involvement from scientists is necessary to avoid such misconceptions and assist in the policy-making process. Developing appropriate regulations, including guidelines, practices, and standards, is important because it will affect how the technology is used globally and facilitate the necessary risk assessment by governments and insurance companies to ensure biotechnology investment, which is a critical component for capitalizing on the promise of cell-free synbio. This raises the question: *Where do cell-free synbio and life-similar systems currently fit in the regulatory landscape?* More importantly, *how does one govern emerging technologies, while ensuring safety and security without preventing global access to the resulting benefits?*

Cell-free systems and the technologies developed to build them might be considered enabling technologies covered under the *seven experiments of concern*, which were identified in 2004 by the National Academies of Science, Engineering, and Medicine. These experiments,

including those that facilitate evasion of being detected, were recommended to undergo “review and discussion by informed members of the scientific and medical community before they are undertaken or published”, due to their impact on biosecurity (*272*). Seventeen years later, the application of cell-free synbio further increased the plausibility and probability of these potential threats; however, this has not been addressed so far.

Currently, there is a critical opportunity to develop appropriate guidelines to reduce the risks associated with their use. If we consider industrial revolutions of the past, it becomes evident that each revolution is more innovative than the previous one and occurs much more rapidly (*273*). In the “age of hyper-innovation,” where disruptive and transformative technologies are developed and adopted quickly, there is less time for the public to adjust. Thus, it is imperative that we make informed decisions during the initial stages of technology development to shape how, for example, cell-free synbio is incorporated into our society. This includes a global strategy to address and manage innovation through regulation, best practices, and training, including conversations with relevant industry sectors, government agencies, and academic institutions. Recognizing both the potential benefits and threats early in the course of development will enable sustainable growth while providing safety and security without stifling innovation.

5.4.1 CORE SECURITY AND SAFETY ACTIONS

Considering the safety and dual-use potential of cell-free systems, it is important to consider ways to identify and counter possible threats and safety concerns, an approach that is likely to be true for any emerging and disruptive technology. **First,** this should include a technology ***co-development initiative*** that aims to create corresponding biotechnology to mitigate against misuse. For example, when creating a novel genetic code, methods and technology tools to experimentally de-code encrypted sequences can be developed in parallel. Bioinformatic tools capable of decoding encrypted sequences can help to identify potential threats that may not be recognized using current screening methods, which aim to prevent the synthesis of known viral or toxic genes (using the List of Human and Animal Pathogens and Toxins for Export Control developed and maintained by the Australia Group (*274*)). Additionally, applying a safety-by-design approach, such as the design of fail-safe or on/off switches for orthogonal synthetic life-similar

systems integrated into the technology development pipeline, would be beneficial to safeguard against their intentional or accidental release. Such technology co-development, mandated by funders, publishers, institutions, and governments will help to encourage scientists throughout the DBTL process to assess and prepare for alternative ways in which their research may be applied and demonstrate to the public proactive risk management.

Second, it is important to develop a global ***technology co-assessment*** culture and provide the required tools to all involved parties. Common practices of technology assessment will be critical for an evidence-based, open and inclusive global conversation about the use of cell-free and life-similar technologies. Given the exponential development speed, it is important to enable and facilitate the co-creation of sustainable regulation at the point of technology inception. The implementation of this hinges on the ability of the participating groups to assess the potential for unintentional use of cell-free and life-similar systems, as well as a foundational ethical framework. The current debate over what is considered “novel biotechnology” has led to some exclusion regarding risk assessment and policy development, further highlighting the need to have these critical conversations. With this in mind, structures need to be established that include technology assessment training and awareness alongside related scientific training, such as in high schools during the initial stages of synbio education (i.e. the International Genetically Engineered Machine competition) (*275*). Society as a whole will be greatly affected, both positively and negatively, by the potential benefits and risks, thus it is necessary for the effective adoption of norms and guidelines around cell-free synbio, that these guidelines are inclusive, and that they can be implemented by emerging economies with less developed regulatory or monitoring systems and institutions.

It is proposed that proactive security and safety measures, including mitigation strategies and surveillance tools, are discussed, co-developed and implemented immediately on a global scale to protect against the misuse of cell-free systems, in contrast to developing regulations once it is too late, especially considering the risk associated with a potential population-wide event. Given the current situation with COVID-19, the economic impact in response to the pandemic potential of a manmade non-lethal virus urges the development of appropriate policies and regulations,

together with the necessary mitigation technologies. These measures are important for realizing the full potential of synbio and corresponding emerging technologies, and to not encounter a situation where advancement of cell-free and life-similar technology is delayed or stopped due to overregulation and lack of social license. Getting this right will not only improve implementation and acceptance of the guiding principles but will also enable leapfrogging into the affected technology space and facilitate the corresponding global sharing of benefits.

5.5 STARTING A GLOBAL REGULATORY CONVERSATION

At both national and international levels, there are a variety of policies that aim to reduce biosecurity risks and minimize biosafety threats. Specific agencies focus on different aspects of developed biotechnology, while the degree of regulation varies extensively between countries (*276*). This results in a complicated and disjointed regulatory framework with the potential for gaps or loopholes. On the part of researchers, this also leads to an administrative burden as they struggle to identify and comply with relevant regulations.

Currently, the Convention on Biological Diversity (CBD) focuses on the impact of synthetic biology including gene editing and gene drive technology at an international scale (*277*), while the U.S. Department of Defense is concerned with bioterrorism in the form of engineered superbugs (*270*), and other agencies discuss the general impact of GMOs. Within these policies, it is unclear where cell-free systems or emerging life-similar technologies fit, and there is no discussion of their potential benefits and risks. While most protocols govern the use of living modified organisms, this term does not cover cell-free and synthetic life-similar systems (*278*). With non-living systems receiving considerably less attention and only mentioned briefly, it is a missed opportunity to proactively assess the risks and benefits of cell-free and life-similar technology.

To amend this lack of inclusion and overcome the current issues of a disjointed regulatory framework, it is suggested that an international organization, such as the World Health Organization, UN, or the CBD, assesses the potential biosecurity risks associated with orthogonal cell-free synbio and life-similar systems. Given that it is not always easy for existing programs within these organizations to adjust their focus in response to the rapidly changing biotechnology

landscape, there is an opportunity to create an independent body to evaluate the biosafety of next-generation synbio. Such a program could be supported by a not-for-profit organization and operates under the umbrella of one of these larger institutions, where it has the potential to complement existing programs by interfacing with related activities (e.g., the UN Biological Weapons Convention, the CBD Cartagena Protocol on Biosafety). This will require involvement from scientists to provide the necessary technical expertise to inform policy development.

The need for global approaches to assess the impact of emerging biotechnologies, such as cell-free and life-similar systems, is recognized by the Nuclear Threat Initiative, Biosecurity Division (NTI:Bio), which convened the Global Biosecurity Dialogue in Addis Ababa in 2019 (supported by the African Union and Global Affairs Canada). NTI:Bio identified emerging biological risks caused by developing technology as one of their major work streams, resulting in the Biosecurity Innovation and Risk Reduction Initiative (BIRRI). Last year they also released a report with the World Economic Forum recommending improved DNA screening procedures to “prevent illicit DNA synthesis and misuse” (*279*). More recently, NTI:Bio and the BIRRI continued discussions virtually building towards the establishment of a global entity focused solely on biosecurity risk reduction. A main priority within this initiative is the creation of standards that will assist funders, grantees, and publishers in the identification of biological risk and improving risk mitigation (*280*). In line with these efforts, it would be beneficial for such an organization to conduct a review of cell-free technologies, assisting in addressing the regulatory challenges that lie ahead. For example, how does one classify a self-replicating artificial cell and what impact does compartmentalization have on regulation? Should cell-free systems be regulated differently depending on their use or application? How does one assess the technology that enables advances with respect to dual-use? How does orthogonality of these systems impact detection and removal of harmful bio-agents produced? Does the enabling technology or end-product belong to its country of origin and how does this impact who has access to the benefits provided by the technology?

5.6 CONCLUSION

As cell-free synbio matures, it is necessary to develop appropriate safety and security measures, while recognizing the exciting new developments made possible by this area of bioengineering. It is our responsibility to encourage global societal discussions on important scientific and technical advances and seek social licensing and consent early in the process. Due to the immense potential of cell-free biotechnology and the likely role it will play in addressing global problems in the future, inclusion in the regulatory conversation will ensure responsible use and preparedness while also fostering innovation.

CHAPTER 6 – FUTURE DIRECTIONS

Research presented here contributes to the advancement and accessibility of cell-free systems for synthetic biologists. Below we discuss ways to build on my research and provide approaches to further improve *in vitro* TX-TL processes and achieve streamlined system preparation. Ultimately, this will enable the use of cell-free systems for biotechnology development.

6.1 TROUBLESHOOTING AND HIGH-THROUGHPUT TESTING

A challenge associated with the in-house preparation of cell-free systems is the identification of components that are sub-optimal or non-functional. To functionally characterize each component, specific assays are required which are time-consuming to set-up and not always feasible to conduct. Alternatively, providing a library of standardized test constructs to assist in the troubleshooting process would be beneficial. For example, in Chapter 2 we demonstrated the utility of a Spinach-tagged EYFP reporter to characterize transcription and translation, which would benefit from additional characterization. Furthermore, the synthesis of specific test peptides coupled with mass spectrometry could be a useful tool, for example, to validate aaRS's (i.e. when the cognate aaRS is functional, the correct amino acid is incorporated into the polypeptide chain and detected via mass spectrometry. Alternatively, if the cognate aaRS is non-functional, the correct amino acid would not be identified.)

In Chapter 3, select factors were identified as potential candidates to improve protein synthesis capacity. Future work aimed at investigating the effect of multiple components, such as those identified in Chapter 3, will benefit from high-throughput screening methods. This will allow for a wide range of reaction conditions to be tested, such as varying component concentrations and testing different combinations of factors. Current methods for high throughput analysis include the use of 96-well plates or microfluidic approaches, which allow for the use of small reaction volumes (*100*).

6.2 IMPROVED RIBOSOME FUNCTIONALITY

A major bottleneck in system performance is poor ribosome functionality resulting in low protein yields. As demonstrated in Figure A4-1, we hypothesize that protein synthesis capacity is limited by a low number of ribosomes actively translating, resulting in few rounds of translation or that only a limited number of ribosomes are capable of initiating translation. Alternatively, the availability of mRNAs could also be limiting. To tease apart which of these factors contribute to a low protein yield, different aspects of *in vitro* gene expression need to be investigated. For example, fluorescent RNA aptamers could be used to monitor mRNA levels, while toeprinting assays could be employed to measure translation initiation. It is important to note that the latter case is challenging to employ as a high-throughput assay. To provide positional information about the location of actively translating ribosomes on the mRNA transcript, using ribosome profiling methods, such as RiboLace (*281*), would be valuable. Furthermore, our research assessed the protein component of ribosomes used for *in vitro* protein synthesis. An investigation of the ribosomal RNA, through RNA extraction and RNA-seq, would provide valuable information about this important component to ribosome function. This also includes examining ribosomal RNA modifications.

6.3 ELUCIDATING THE FUNCTIONAL RELEVANCE OF RIBOSOMAL PROTEIN S1

Chapter 4 demonstrated the benefit of supplementing ribosomal protein S1 to an *in vitro* reconstituted cell-free reaction resulting in increased protein synthesis capacity. It was hypothesized that S1 could bind to ribosomes improving mRNA recruitment, and subsequently translation initiation, or unwinding structured RNA's facilitating ribosome binding to the mRNA and improving mRNA stability. To better understand the function of S1 additional studies are needed. For example, to determine if S1 has an effect on T7 RNA polymerase, mRNA could be directly added to the system or a dual TX-TL reporter, such as Spinach-tagged EYFP, could be employed to deconvolute the effect of S1 on transcription and translation. Regarding the role of S1 in aiding translation initiation, toeprinting assays are a common technique used to study the translation initiation complex and elucidate the importance of structural elements or associated factors (*255,*

282). Furthermore, to help understand S1's mechanism of action, varying the genetic context to include other structured 5'UTRs and/or codon usage would be beneficial.

6.4 SIMPLIFIED TRNA PURIFICATION

Preparing *in vitro* reconstituted TX-TL systems in-house provides benefits of increased control over the system composition allowing for modular and engineerable design, as well as providing a cost-effective alternative to commercial systems. A highly functional energy solution is required for efficient translation. One of the more expensive components with limited modularity is the tRNA component, which is typically purchased commercially, or extracted from cells, as total tRNA. A simple method to purify individual tRNA isoacceptors is needed and would allow for customized tRNA compositions, with increased control for non-canonical amino acid incorporation.

6.5 NOVEL DEPLOYMENT STRATEGIES

To expand on the applications and uses of cell-free systems, new deployment strategies are required that provide novel design features not available with current systems. An underdeveloped method, touched on in Section 1.6, is a programmable RNA hydrogel. Hydrogels are hydrophilic polymer structures that have properties similar to both liquids and elastomers. They are generally made from synthetic or natural polymers, including DNA, proteins, and carbohydrates. Alternatively, RNA hydrogels are much more recent and largely unexplored. The use of RNA has the potential to provide unique features, such as programmability (i.e. can be transcribed from a DNA template), structural flexibility, and the ability to produce protein directly from the hydrogel. Previous work has demonstrated controlled functionalization of protein- and DNA-based hydrogels for applications in drug delivery and synthetic biology. We hypothesize RNA hydrogels can be functionalized in a similar manner.

Preliminary work was conducted by incorporating anti-his aptamers into a previously studied RNA hydrogel that has been shown to form via self-assembly (*115*). Functionalization with select aptamers transforms the hydrogel into a protein scaffold that can be used for the controlled execution of *in vitro* reaction cascades. The first step requires demonstration of his-tagged protein (e.g., GFP) binding to the anti-his aptamers of the hydrogel. As a secondary proof of concept, the

binding of functional his-tagged EF-G to the hydrogel and measuring GTP hydrolysis would demonstrate functional enzyme activity. Such work would add to the repertoire of hydrogels, expanding their potential uses and applications.

6.6 MONITORING THE REGULATORY LANDSCAPE OF NEXT-GENERATION SYNTHETIC BIOLOGY

Next-generation synthetic biology and the associated technology is rapidly changing and expanding. Therefore, it is important to follow-up and assess how current policy and regulations adapt with this change. Expanding on the discussion points covered in Chapter 5, there is a growing need for a cell-free consortium, analogous to the International Gene Synthesis Consortium, that can safeguard the growing commercialization of cell-free systems. For example, start-up companies that utilize and develop cell-free systems require a place to consult about biosecurity and biosafety measures in regard to their technology. How the commercialization of cell-free systems is handled is an important piece to the safe and responsible use of next-generation synbio, while also promoting innovation that produces tangible benefits to society.

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APPENDIX 1 – SUPPLEMENTAL MATERIAL TO CHAPTER 1

Table A1-1: Function of each component in the PURE system (*283, 284*)

	Protein	Function
Solution A	Amino Acids	Substrates for protein synthesis; building blocks of proteins
	Magnesium Acetate	Mg^{2+} is an important cofactor for activity of enzymes
	Potassium Glutamate	Involved in protein stabilization
	Dithiothreitol (DTT)	Prevents the formation of disulfide bonds, which may inactivate catalysts (*50, 285*)
	Adenosine triphosphate (ATP)	Substrate for transcription; energy currency
	Guanosine triphosphate (GTP)	Substrate for transcription; energy currency
	Cytidine triphosphate (CTP)	Substrate for transcription
	Uridine triphosphate (UTP)	Substrate for transcription
	Transfer RNA (tRNA)	Delivers an amino acid to the translation machinery; involved in decoding the mRNA sequence into protein
	Creatine Phosphate	Energy currency; high energy phosphate bond donor
	Folinic Acid	Stimulates the initiation of translation by providing the precursor for formyl methionine formation
	Spermidine	Involved in nucleic acid stabilization
Solution B	Initiation Factor 1 (IF1)	Associates with the 30S subunit in the A-site; prevents an aminoacyl-tRNA from entering (aa-tRNA); modulates IF2 binding to the ribosome by increasing its affinity
	Initiation Factor 2 (IF2)	GTPase; promotes binding of the initiator fMet-$tRNA^{fMet}$ to the 30S subunit
	Initiation Factor 3 (IF3)	Binds to the 30S subunit; blocks the 50S subunit from binding to allow for the pre-initiation complex to form
	Elongation Factor G (EF-G)	GTPase; catalyzes translocation of tRNA and mRNA through the ribosome
	Elongation Factor Thermo Unstable (EF-Tu)	GTPase; catalyzes the binding of aa-tRNA to the ribosome; facilitates the selection and binding of aa-tRNA to the A-site of the ribosome
	Elongation Factor Thermo Stable (EF-Ts)	Guanine nucleotide exchange factor; catalyzes the release of GDP from EF-Tu
	Release Factor 1 (RF1)	Helps the ribosome to dissociate form the mRNA; recognizes the termination codons UAA and UAG;
	Release Factor 2 (RF2)	Helps the ribosome to dissociate form the mRNA; recognizes the termination codons UAA and UGA;
	Release Factor 3 (RF3)	GTP-binding protein; leads to the dissociation of RF1 and RF2 after peptide release
	Methionyl tRNA formyltransferase (MTF)	Attaches a formyl group to the free amino group of methionyl-tRNA
	Ribosome Recycling Factor (RRF)	Splits ribosomal subunits; releases bound mRNA
	Myokinse (MK)	Phosphotransferase; catalyzes the interconversion of adenine nucleotides (ATP, ADP, AMP)
	Creatine Kinase (CK)	Regenerates ATP from ADP; creatine phosphate is the substrate
	Nucleotide Diphosphate Kinase (NDK)	Catalyzes the exchange of terminal phosphate between different nucleoside diphosphates (NDP) and triphosphates (NTP)
	Inorganic Pyrophosphatase (PPiase)	Catalyzes the conversion of one molecule of pyrophosphate to two phosphate ions
	T7 RNA Polymerase (T7 RNAP)	Transcription factor from T7 bacteriophage; transcribes DNA downstream of a T7 promoter
	Aminoacyl-tRNA Synthetases (aaRS)	Attaches the appropriate amino acid to the cognate tRNA
	Ribosomes	Macromolecular machine composed of protein and RNA; decodes mRNA; synthesizes proteins

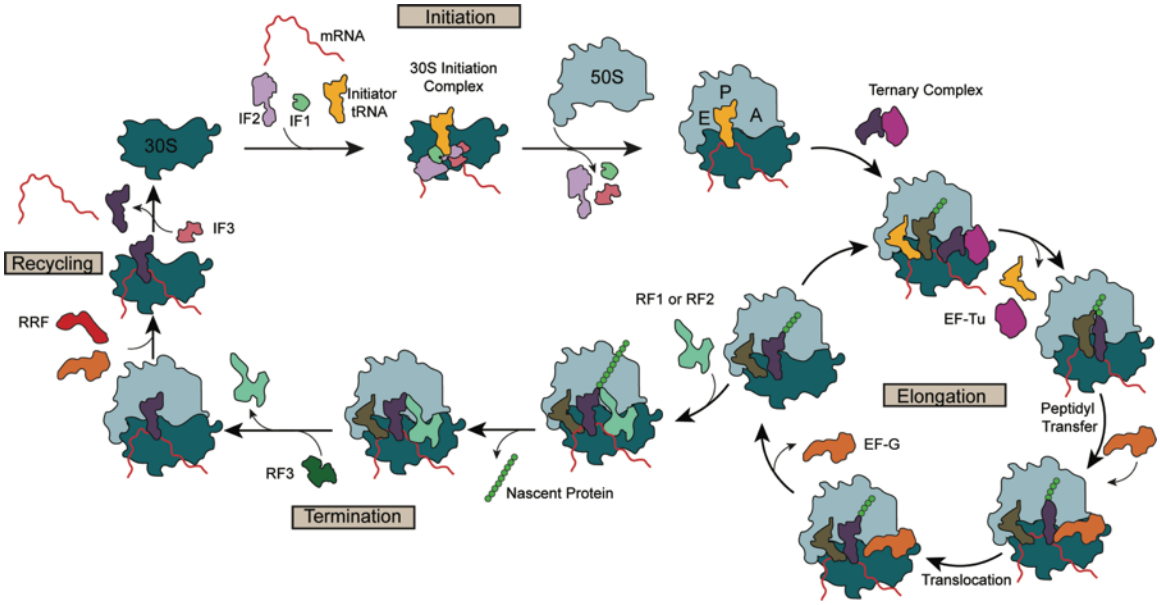

Figure A1-1: Overview of translation in *E. coli*.

The ribosome, with the help of associated factors, decode mRNA to form a polypeptide sequence in a process called translation. This process consists of four main steps: 1) initiation, 2) elongation, 3) termination, and 4) recycling (*284*). Canonical translation initiation involves binding of the small ribosomal subunit (30S) to a specific sequence of the mRNA called a Shine Dalgarno (SD) sequence. The SD is approximately 8-10 nucleotides upstream of the start codon and interacts with the anti-SD sequence of the 16S rRNA in the 30S subunit (*284*). Translation initiation is mediated by three initiation factors: IF1 binds to the A-site of the 30S subunit blocking an aminoacyl-tRNA from entering the A-site and modulates IF2 and IF3 activity; IF2 is a GTPase that helps position the fMet-tRNA^{fMet} into the P-site; IF3 prevents association of the 50S subunit (*284*). The 30S ribosomal subunit, IF1, IF2, IF3, and fMet-tRNAfMet form the 30S preinitiation complex. Upon mRNA recruitment and recognition of the start codon, the complex is stabilized resulting in the 30S initiation complex (*284*). Binding of the large 50S ribosomal subunit causes the initiation factors to dissociate and the fMet-tRNA^{fMet} to accommodate into the P-site forming the 70S initiation complex (*284*). Translation elongation follows, where the cognate aminoacyl-tRNA recognizes the codon in the A-site. Delivery of an aminoacyl-tRNA to the ribosome is provided in a ternary complex with EF-Tu and GTP. Codon anti-codon interaction triggers GTP hydrolysis in EF-Tu followed by the

release of the formed P_i and a conformational rearrangement of the protein. The change in conformation results in the release of aminoacyl-tRNA into the A-site followed by GDP bound EF-Tu dissociation (*284*). Peptide bond formation occurs in the ribosomes peptidyl transferase center between the peptidyl- and aminoacyl-tRNA in the P and A site, respectively. To allow subsequent rounds of decoding and peptide bond formation, EF-G promotes translocation or movement of tRNA and mRNA through the ribosome (i.e. P-site tRNA moves to the E-site and the A-site peptidyl-tRNA moves to the P-site freeing the A-site for the incorporation of the next aminoacyl-tRNA) (*284*). The elongation process stops when the ribosome encounters a stop codon, prompting hydrolysis of the peptidyl-tRNA ester bond and release of the polypeptide. Translation termination is facilitated by RF1 or RF2, where RF1 recognizes the stop codon UAG/UAA and RF2 recognizes the stop codon UGA/UAA. A third factor, RF3, helps RF1 and RF2 to dissociate from the ribosome. In order to repeat the process of peptide synthesis and initiate subsequent rounds of translation, the ribosome must dissociate into the two subunits (50S and 30S) (*284*). Ribosome recycling factor and EF-G catalyze this process of ribosome splitting and release bound mRNA and tRNA, freeing the subunits for continued cycles of translation (*284*).

APPENDIX 2 – SUPPLEMENTAL MATERIAL TO CHAPTER 2

Supplementary Tables

Table A2-1: In-house energy solution composition

Component	Catalog Number	Company	Stock Concentration	4X Sub-stock Concentration	Final Concentration	Units
Amino Acids	LAA21-1KT	Sigma-Aldrich	12	1.2	0.3	mM
Magnesium Acetate	M0631	Sigma-Aldrich	1000	47.2	11.8	mM
Potassium Glutamate	49601	Sigma-Aldrich	2000	400	100	mM
DTT	BP172	Fischer Scientific	500	4	1	mM
ATP	A26209	Sigma-Aldrich	100	8	2	mM
GTP	g8877	Sigma-Aldrich	100	8	2	mM
CTP	C1506	Sigma-Aldrich	100	4	1	mM
UTP	U6750	Sigma-Aldrich	100	4	1	mM
tRNA	10109550001	Roche	1900	208	52	U A_{260}/mL
Creatine Phosphate	27920	Sigma-Aldrich	500	80	20	mM
Folinic Acid	AC230312500	Acros Organics	1000	40	10	µg/mL
Spermidine	S2626	Sigma-Aldrich	500	8	2	mM

Table A2-2: Protein purification buffer composition

Component	Catalog Number	Company	Buffer A	Buffer B	Buffer E	Storage Buffer ($TAKM_7$)
Tris Base	BP152-5	Fisher Scientific	50 mM	50 mM	50 mM	50 mM
Ammonium Chloride	A661-500	Fisher Scientific	60 mM	60 mM	60 mM	70 mM
Magnesium Chloride	M35-500	Fisher Scientific	7 mM	7 mM	7 mM	7 mM
Potassium Chloride	P217-500	Fisher Scientific	300 mM	300 mM	300 mM	30 mM
Imidazole	IB0277	BioBasic	10 mM	20 mM	250 mM	
Glycerol	BP229-1	Fisher Scientific	15 % (v/v)	15 % (v/v)	15 % (v/v)	
β-mercaptoethanol	6050	Millipore	7 mM	7 mM	7 mM	
PMSF	PB0452	BioBasic	1 mM	1 mM	1 mM	
pH			8.0	8.0	8.0	7.5

Table A2-3: Ribosome purification buffer composition - step 1

Component	Catalog Number	Company	Cell Opening	Sucrose Cushion	Washing Buffer	Overlay Buffer	Intermediate Storage Buffer
Tris Base	BP152-5	Fisher Scientific	20 mM	20 mM	20 mM	20 mM	20 mM
Ammonium Chloride	A661-500	Fisher Scientific	100 mM	500 mM	500 mM	60 mM	60 mM
Magnesium Chloride	M35-500	Fisher Scientific	10.5 mM	10.5 mM	10.5 mM		
Magnesium Acetate	BP215-500	Fisher Scientific				5.25 mM	5.25 mM
EDTA	BP118-500	Fisher Scientific	0.5 mM	0.5 mM	0.5 mM	0.25 mM	0.25 mM
β-mercaptoethanol	6050-250	Millipore	3 mM	3 mM	7 mM	3 mM	3 mM
D-Sucrose	BP220-1	Fisher Scientific		1.1 mM			5% (w/v)
pH			7.6	7.6	7.6	7.6	7.7

Table A2-4: Ribosome purification buffer composition - step 2

Component	Catalog Number	Company	Overlay Buffer	50% Sugar	10% Sucrose	40% Sucrose	50% Sucrose	Storage Buffer
Tris Base	BP152-5	Fisher Scientific	20 mM		20 mM	20 mM	20 mM	50 mM
Ammonium Chloride	A661-500	Fisher Scientific	60 mM		60 mM	60 mM	60 mM	30 mM
Magnesium Chloride	M35-500	Fisher Scientific						5 mM
Magnesium Acetate	BP215-500	Fisher Scientific	5.25 mM		5.25 mM	5.25 mM	5.25 mM	
EDTA	BP118-500	Fisher Scientific	0.25 mM		0.25 mM	0.25 mM	0.25 mM	
β-mercaptoethanol	6050-250ml	Millipore	3 mM		3 mM	3 mM	3 mM	
D-Sucrose	BP220-1	Fisher Scientific			10% (w/v)	40% (w/v)	40% (w/v)	
Potassium Chloride	P217-500	Fisher Scientific						30 mM
Sugar		Rogers		50% (w/v)				
pH			7.6	7.6	7.6	7.6	7.6	7.6

Table A2-5: Additional reagents for protein and ribosome purification

	Component	Catalog Number	Company
LB Media	Yeast extract	G0961	BioBasic
	Tryptone	G217(G211)	BioBasic
	Table Salt	N/A	Generic Brand
Antibiotics	Kanamycin	KB0286	BioBasic
	Chloramphenicol	CB0118	BioBasic
	Ampicillin	AB0028	BioBasic
Cell Opening	Sodium Deoxycholate	DD0150	BioBasic
	Lysozyme	LDB0308	BioBasic
	DNase I	d4513-1vl	SigmaAldrich
	Aluminum oxide	265497-2.5kg	SigmaAldrich

Table A2-6: TX-TL protein plasmid details

Protein	DNA Sequence	Organism	Vector	Antibiotic	Strain
AlaRS	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCATCATCATCATCATGGTGGTAGCGGTGGTGGTAGCGGTTCCAA GAGTACCGCGGAGATCCGCCAAGCCTTTTTGGACTTTTTCCACTCCAAGGGTCATCAAGTTGTGGCATCATCCAGCTTGGTGCCTCACAACGACCCTACGCTGTTATTCACCAATGCCGGTA TGAACCAATTCAAGGATGTGTTTTTGGGGTTGGACAAACGCAACTATTCTCGCGCAACCACATCACAACGCTGTGTCCGCGCAGGAGGTAAGCATAATGATTTGGAGAATGTCGGCTACACG GCGCGTCACCACACATTTTTCGAGATGCTTGGGAACTTTTCGTTTGGAGACTACTTTAAACACGACGCGATTCAGTTCGCATGGGAGCTGCTGACCTCCGAGAAGTGGTTCGCACTGCCTAA AGAACGCTTATGGGTTACTGTGTATGAATCGGACGACGAAGCTTATGAGATCTGGGAGAAGGAAGTAGGTATCCCACGCGAACGCATCATCCGCATCGGCGATAACAAGGGGGCCCCCTAT GCATCTGACAATTTCTGGCAAATGGGAGATACCGGCCCGTGTGGGCCTTGTACTGAAATTTTTTACGATCATGGCGACCACATCTGGGGGGGGCCTCCTGGATCCCCTGAAGAAGATGGTG ATCGCTACATCGAGATCTGGAACATTGTGTTCATGCAGTTCAACCGTCAGGCAGACGGGACTATGGAGCCCCTGCCAAAGCCATCTGTTGATACGGGAATGGGATTGGAGCGCATTGCGGC TGTTTTACAGCACGTCAACTCAAACTACGATATCGATTTATTCCGCACTCTGATCCAGGCGGTGGCAAAAGTAACGGGAGCAACGGACCTGAGCAACAAGTCCCTGCGTGTTATCGCCGATC ATATTCGTTCATGTGCGTTTCTTATTGCTGACGGAGTGATGCCATCCAACGAAAATCGCGGATATGTCCTTCGTCGTATCATTCGTCGTGCCGTGCGCCACGGAAATATGTTGGGTGCCAAA GAGACCTTCTTTTATAAGTTAGTGGGTCCTCTTATTGATGTCATGGGGTCCGCCGGCGAAGACTTAAAGCGTCAACAGGCCCAGGTAGAACAAGTGTTAAAGACCGAAGAGGAGCAATTCG CCCGCACGCTGGAACGTGGGCTGGCCCTGCTTGATGAAGAATTGGCCAAGCTGAGTGGGGATACATTAGATGGGGAGACAGCCTTCCGCCTTTATGACACGTACGGTTTCCCTGTTGACTT GACGGCGGACGTGTGCCGTGAACGCAATATCAAGGTGGACGAAGCCGGGTTTGAAGCCGCTATGGAAGAACAGCGTCGCCGTGCCCGCGAAGCGTCTGGGTTTGGCGCTGACTATAATG CCATGATTCGCGTCGATTCGGCCTCAGAGTTCAAGGGGTACGACCATCTTGAGTTAAACGGGAAGGTCACAGCGTTATTCGTGGATGGGAAGGCGGTAGACGCGATTAACGCCGGACAGG AAGCTGTCGTGGTCTTGGATCAAACGCCTTTTTATGCCGAGAGTGGTGGCCAGGTAGGTGATAAAGGTGAATTGAAGGGGGCGAATTTCTCCTTCGCGGTGGAGGACACGCAGAAATATGG GCAAGCTATCGGACACATCGGCAAATTGGCTGCTGGATCGCTTAAAGTCGGCGACGCTGTCCAGGCGGACGTTGACGAGGCGCGTCGTGCGCGTATCCGCTTAAACCATTCAGCAACCCA CCTTATGCATGCAGCTCTTCGTCAGGTCTTAGGCACACATGTCTCCCAAAAAGGTTCCTTGGTTAACGATAAGGTTCTGCGCTTCGACTTCTCGCATAACGAAGCTATGAAACCGGAAGAGA TTCGCGCGGTTGAGGACTTAGTCAATACGCAGATTCGTCGTAATTTGCCAATCGAAACAAATATCATGGACCTGGAGGCCGCCAAGGCTAAGGGGGCTATGGCCTTATTTGGCGAGAAGTA CGACGAACGCGTCCGCGTCCTTTCAATGGGTGACTTCAGTACAGAATTGTGCGGTGGCACGCACGCTTCTCGTACGGGTGACATTGGCTTATTCCGTATCATTTCGGAGAGCGGCACAGCC GCAGGGGTTCGCCGCATTGAAGCGGTGACAGGGGAGGGAGCCATCGCTACTGTACATGCTGACTCTGATCGCCTTTCTGAAGTTGCTCACTTGCTGAAAGGAGACAGCAACAACTTAGCG GATAAAGTACGCAGTGTACTGGAACGTACGCGTCAGCTTGAAAAAGAGTTGCAGCAATTAAAGGAGCAGGCAGCCGCACAGGAGAGTGCAAACTTATCAAGCAAGGCCATCGATGTCAATG GAGTCAAGTTACTTGTTTCAGAATTATCTGGTGTGGAGCCCAAAATGCTGCGCACCATGGTCGATGACCTGAAAAATCAATTGGGTTCCACCATCATCGTACTTGCGACGGTGGTAGAAGGC AAGGTCTCCCTGATTGCAGGTGTATCTAAAGATGTAACTGATCGCGTAAAGGCGGGCGAATTGATTGGTATGGTCGCCCAACAGGTAGGAGGGAAGGGCGGGGGGCGTCCCGACATGGCT CAAGCTGGTGGTACTGACGCGGCAGCACTGCCCGCTGCATTGGCCTCCGTGAAAGGATGGGTCTCGGCTAAATTGCAATAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAG TCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG - 3'	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
ArgRS	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCATCATCATCATCATGGTGGTAGCGGTGGTGGTAGCGGTAACATC CAGGCATTGTTATCCGAGAAAGTCCGCCAAGCCATGATTGCCGCTGGTGCACCCGCGGATTGTGAACCGCAAGTACGTCAGTCAGCTAAAGTTCAATTCGGCGATTACCAAGCCAACGGCA TGATGGCTGTCGCGAAGAAGTTGGGCATGGCACCTCGCCAGTTGGCGGAACAAGTTCTTACACACTTGGACTTAAATGGCATTGCGAGCAAGGTGGAGATTGCGGGACCTGGTTTCATTAA CATTTTTTTAGACCCCGCATTCTTAGCGGAACATGTGCAGCAGGCCTTGGCTTCGGACCGCTTGGGAGTAGCTACGCCTGAAAAGCAGACTATCGTAGTAGACTATTCGGCCCCTAACGTG GCAAAGGAGATGCACGTGGGGCACTTGCGCTCAACCATTATTGGTGACGCCGCCGTTCGTACCTTGGAATTTTTAGGCCATAAAGTAATTCGCGCAAATCACGTCGGAGACTGGGGTACGC AATTCGGTATGTTAATCGCTTGGTTAGAGAAGCAGCAGCAAGAGAATGCAGGTGAAATGGAGTTGGCTGATCTTGAGGGTTTTTATCGTGACGCGAAGAAGCACTATGATGAAGATGAGGAG TTTGCAGAACGCGCGCGTAATTACGTCGTGAAACTTCAAAGTGGCGACGAGTATTTCCGCGAAATGTGGCGTAAGCTGGTGGATATTACTATGACGCAGAACCAGATCACTTATGACCGCTT GAATGTCACTTTAACCCGCGATGATGTCATGGGCGAATCTCTGTATAAATCCTATGCTTCCAGGAATTGTGGCAGATTTGAAGGCAAAAGGCCTTGCCGTCGAGAGTGAGGGCGCCACGGTG GTATTTCTTGATGAGTTTAAGAATAAGGAAGGTGAACCCATGGGGGTTATCATCCAGAAGAAAGACGGAGGTTACCTTTACACCACGACTGACATTGCCTGTGCAAAATATCGCTATGAAACA CTTCACGCGGACCGTGTTCTTTACTATATCGATTCCCGTCAACATCAACATCTGATGCAAGCATGGGCGATTGTGCGTAAGGCTGGATACGTCCCAGAATCCGTGCCGTTGGAGCATCATAT GTTCGGCATGATGCTGGGAAAGGATGGAAAACCCTTCAAAACCCGTGCAGGGGGAACAGTTAAACTTGCCGATTTGTTGGACGAGGCGCTGGAGCGTGCGCGTCGCTTAGTTGCAGAAAA AAATCCTGATATGCCCGCAGACGAGTTGGAAAAGCTGGCAAACGCTGTCGGAATTGGGGCAGTCAAATATGCCGATCTTTCCAAAAACCGCACCACGGACTACATTTTCGACTGGGATAACA TGCTGGCGTTCGAAGGGAACACAGCTCCTTACATGCAATATGCCTACACGCGTGTTCTGTCTGTTTTCCGCAAAGCCGAAATCGACGAAGAGCAGCTTGCTGCTGCCCCGGTTATCATCCG CGAGGATCGTGAGGCCCAACTGGCGGCACGCCTGTTACAGTTTGAAAGAGACTTTGACAGTTGTCGCCCGTGAAGGAACACCCCACGTAATGTGTGCTTACCTTTACGACTTGGCTGGGCTT TTTTCTGGGTTCTACGAGCACTGTCCAATCTTATCCGCGGAAAATGAGGAGGTTCGCAACTCCCGTCTGAAGTTGGCCCAATTGACTGCGAAGACATTGAAACTGGGATTAGACACTCTTGG TATTGAGACCGTTGAGCGTATGTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGA GTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
AsnRS	5'- GAATTCGGCCGCTTAGAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCATCATCATCATCATGGTGGTAGCGGTGGTGGTAGCGGTTCTGTGGT TCCAGTGGCTGATGTGCTTCAAGGACGCGTGGCAGTCGATTCCGAGGTGACGGTTCGCGGATGGGTTCGCACGCGTCGTGATTCCAAGGCAGGAATCAGTTTTCTTGCAGTGTATGACGG TTCTTGTTTTGACCCTGTACAGGCAGTGATTAATAACAGTTTGCCGAACTACAACGAAGATGTCTTGCGTTTAACTACCGGCTGTTCCGTAATCGTAACGGGAAAGGTAGTGGCCAGCCCCG GTCAGGGACAACAGTTTGAGATTCAGGCATCCAAGGTGGAAGTCGCTGGGTGGGTTGAAGACCCAGACACGTATCCCATGGCTGCAAAACGCCATTCAATTGAATATCTGCGCGAAGTGGC GCATTTACGCCCGCGCACTAACCTGATCGGGGCTGTGGCACGCGTACGTCATACCTTAGCTCAAGCCCTGCACCGTTTTTTCAACGAACAAGGGTTCTTTTGGGTTTCGACTCCGCTGATTG CGGCATCTGATACCGAAGGAGCGGGCGAGATGTTCCGTGTTTCGACACTGGACTTGGAAAACCTTCCCCGCAACGATCAGGGAAAGGTCGATTTTGATAAAGATTTTTTCGGCAAAGAATCA TTCCTGACTGTGTCCGGGCAACTTAATGGCGAGACTTACGCGTGTGCTTTGTCCAAGATTTATACGTTCGGACCAACATTCCGCGCGGAAAACTCGAACACAAGTCGTCATCTTGCCGAATT TTGGATGTTGGAACCAGAAGTGGCTTTTGCTAACTTGAACGATATTGCAGGGTTGGCTGAGGCAATGTTAAAATACGTTTTCAAGGCAGTTTTGGAGGAACGTGCGGATGATATGAAGTTCTT CGCCGAGCGCGTAGACAAAGATGCCGTTTCACGCTTAGAACGTTTTATCGAAGCCGATTTTGCACAGGTGGATTATACCGATGCCGTAACTATTTTGGAAAACTGCGGCCGCAAATTTGAAA ACCCGGTTTATTGGGGCGTAGACCTTAGTAGTGAACACGAGCGCTATTTGGCGGAGGAGCACTTTAAAGCTCCCGTAGTTGTGAAGAACTACCCGAAAGACATCAAAGCATTCTACATGCG CTTAAACGAAGACGGTAAGACGGTGGCTGCAATGGACGTTTTGGCCCCAGGAATTGGTGAGATCATTGGGGGCTCACAACGTGAGGAGCGTTTGGATGTTTTGGATGAGCGCATGTTGGAA	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)

	ATGGGCCTTAACAAAGAGGACTATTGGTGGTATCGTGACTTGCGCCGCTATGGTACGGTTCCCCACTCAGGCTTCGGGCTGGGGTTTGAACGTTTGATTGCCTACGTCACGGGAGTCCAAA ACGTCCGCGATGTCATCCCATTCCCGCGTACGCCACGCAACGCGTCATTCTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTG TTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'				
AspRS	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCGTACTGAATATTGTGGTCAACTTCGCTTAAGTCACGTGGGCCAGCAG GTTACTCTGTGCGGCTGGGTGAACCGCCGCCGCGATCTTGGGTCGTTGATTTTTATCGACATGCGTGATCGTGAGGGAATTGTCCAGGTCTTTTTTGATCCGGACCGTGCCGACGCCCTTA AGCTTGCGTCAGAGTTACGCAACGAATTTTGCATCCAGGTTACTGGAACGGTCCGTGCACGTGATGAAAAGAACATTAATCGTGACATGGCGACTGGAGAGATTGAGGTCCTTGCATCAAGT CTTACTATTATTAACCGTGCGGATGTTTTGCCCCCTGGACTCTAACCACGTTAACACTGAAGAAGCCCGTCTTAAGTATCGCTATCTGGACTTGCGTCGCCCGGAAATGGCTCAGCGTTTAAAG ACGCGTGCAAAAATCACATCGCTTGTGCGCCGCTTCATGGATGACCACGGTTTCCTTGATATCGAAACGCCGATGCTTACAAAGGCTACTCCGGAGGGTGCCCGCGACTATCTTGTGCCTT CGCGCGTGCACAAAGGGAAATTCTATGCATTGCCCCAATCCCCCCAACTGTTCAAACAACTTTTAATGATGAGCGGGTTCGACCGCTACTACCAGATTGTTTTCGTGACGAAGAC CTGCGCGCGGATCGCCAACCTGAGTTTACTCAGATCGATGTCGAGACCTCTTTTATGACCGCACCACAAGTACGCGAAGTTATGGAAGCATTAGTACGTCACCTTTGGCTTGAAGTAAAAGG CGTAGATCTGGGAGACTTCCCCGTTATGACTTTTGCCGAGGCGGAACGTCGCTACGGGTCGGACAAACCTGATTTGCGTAATCCAATGGAATTGACTGATGTAGCAGACTTATTAAAATCTG TTGAATTTGCGGTGTTTGCGGGACCTGCAAATGATCCCAAGGGTCGCGTCGCTGCGCTGCGCGTTCCCGGCGGGGCATCGCTTACTCGTAAACAGATTGATGAGTACGGGAACTTTGTGAA GATTTATGGGGCAAAAGGGTTGGCATATATTAAGGTGAACGAGCGCGCGAAAGGCTTAGAAGGCATCAATTCACCAGTAGCTAAGTTCTTGAACGCAGAGATTATCGAAGCTATCCTTGACC GTACGGCAGCACAAGACGGCGATATGATTTTTTTTGGGGCTGACAACAAAAAAATTGTTGCTGACGCAATGGGAGCGTTACGCTTGAAAGTGGGGAAAGACTTGGGATTAACCGACGAGTC TAAATGGGCCCCTTTATGGGTAATCGACTTCCCAATGTTTGAGGATGACGGCGAAGGTGGTCTTACAGCAATGCATCACCCTTTTACCTCTCCTAAAGATATGACTGCCGCCGAATTAAAGG CCGCACCCGAGAACGCCGTGGCTAATGCTTATGATATGGTTATCAATGGATATGAGGTAGGTGGTGGAAGCGTGCGCATTCACAACGGAGATATGCAACAAACAGTTTTCGGCATCCTGGG AATCAATGAAGAAGAACAACGTGAGAAATTCGGCTTTTTGCTGGATGCTTTGAAATATGGCACGCCACCCCATGCCGGTTTGGCCTTTGGATTAGATCGTTTAACTATGTTATTGACAGGTAC GGACAATATCCGCGACGTGATCGCTTTTCCGAAGACTACAGCGGCTGCTTGTCTGATGACCGAAGCACCATCATTCGCAAACCCTACTGCACTTGCAGAGTTGTCAATCCAGGTGGTAAAGA AAGCCGAGAACAATGGTGGTAGCGGTGGTGGTAGCGGTCATCATCATCATCATTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTT TATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
CysRS	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGTTAAAGATTTTCAACACGTTAACACGCCAGAAAGAAGAGTTCAAGCCC ATTCACGCGGGAGAAGTGGGTATGTACGTCTGTGGCATCACAGTGTACGACCTGTGTCATATCGGCCATGGTCGTACGTTCGTAGCATTCGACGTAGTCGCTCGTTATTTACGTTTCTTAGG CTATAAGTTGAAGTATGTTCGCAACATCACGGACATTGACGATAAAATCATCAAACGTGCCAACGAAAACGGAGAGTCGTTCGTTGCTATGGTGGATCGCATGATTGCGGAAATGCATAAGG ATTTCGACGCGCTGAATATCTTGCGCCCCGACATGGAGCCGCGTGCGACTCATCATATTGCCGAAATTATCGAATTAACAGAGCAACTGATTGCTAAGGGGCACGCCTACGTGGCCGACAA CGGTGACGTGATGTTCGACGTGCCGACCGACCCGACTTATGGAGTTTTGTCCCGTCAAGACCTGGACCAGTTGCAAGCTGGGGCGCGCGTCGACGTCGTTGACGATAAACGCAACCCAAT GGACTTCGTCTTATGGAAAATGTCGAAGGAGGGAGAACCATCCTGGCCTAGTCCTTGGGGGGCTGGCCGTCCAGGGTGGCATATTGAGTGCTCGGCAATGAACTGCAAACAGCTTGGAAA TCACTTCGACATCCACGGAGGCGGTTCAGACTTGATGTTCCCCCACCACGAAAATGAGATTGCTCAGAGTACGTGTGCGCACGATGGGCAATATGTAAATTACTGGATGCATTCCGGAATG GTTATGGTAGACCGTGAAAAGATGTCAAAATCGCTGGGCAACTTCTTCACAGTGCGCGACGTACTTAAGTACTATGACGCGGAGACCGTCCGCTATTTTTTAATGAGCGGTCACTACCGCTC ACAGCTTAACTATTCCGAGGAGAACCTGAAACAGGCTCGTGCAGCATTAGAACGCTTGTATACTGCCCTTCGCGGCACAGACAAAACGGTGGCGCCGGCTGGTGGTGAGGCGTTTGAAGC CCGTTTCATCGAAGCTATGGATGACGATTTCAACACCCCGGAGGCTTACTCGGTGTTATTTGACATGGCTCGCGAGGTGAACCGTCTGAAGGCGGAAGATATGGCTGCGGCAAACGCGATG GCAAGCCACTTGCGCAAGCTGTCCGCAGTTTTGGGCCTTTTAGAGCAGGAGCCAGAAGCTTTTCTTCAAAGTGGGGCTCAGGCAGATGACTCGGAGGTAGCCGAAATCGAAGCCCTGATTC AGCAGCGTTTGGATGCTCGCAAGGCTAAGGATTGGGCTGCCGCTGACGCCGCCCGTGATCGTTTAAATGAGATGGGAATTGTCCTGGAAGACGGTCCACAGGGGACAACATGGCGCCGTA AGGGTGGTAGCGGTGGTGGTAGCGGTCATCATCATCATCATTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTG TCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
GlnRS	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCACCATGGTCACCATCACGGTGGTAGCGGTGGTGGTAGCGGTTC AGAGGCCGAAGCTCGCCCTACAAATTTCATTCGTCAGATCATCGACGAGGATCTGGCGAGTGGTAAACACACCACGGTGCATACACGCTTTCCTCCAGAACCTAATGGGTATCTGCATATCG GCCACGCAAAATCCATTTGTTTGAATTTTGGAATTGCCCAGGATTACAAAGGGCAGTGCAACCTTCGCTTTGACGACACTAACCCCGTAAAAGAAGACATTGAGTATGTCGAGTCGATTAAGA ACGATGTTGAGTGGTTAGGCTTTCATTGGTCAGGAAACGTCCGCTACTCAAGTGACTACTTTGACCAACTTCACGCGTACGCAATCGAACTTATCAACAAAGGGTTGGCTTATGTTGACGAG CTGACACCTGAGCAGATCCGTGAATACCGCGGGACGTTGACTCAACCAGGAAAAAACTCGCCATACCGCGACCGTTCGGTCGAGGAGAATCTTGCGTTATTCGAAAAGATGCGTGCGGGC GGTTTTGAGGAGGGGAAAGCTTGTCTGCGCGCGAAAATTGATATGGCCTCCCCATTTATTGTTATGCGTGACCCTGTCCTGTACCGCATCAAATTCGCTGAGCATCATCAAACCGGGAACAA ATGGTGTATTTACCCAATGTACGACTTCACACACTGTATCTCCGATGCCCTGGAGGGCATTACCCACTCGTTGTGCACGTTAGAGTTCCAAGACAATCGTCGTCTTTATGATTGGGTCCTTGA TAATATCACCATCCCGGTACATCCCCGTCAATACGAGTTTTCGCGTTTAAATCTGGAATATACTGTGATGTCGAAGCGTAAACTGAATCTTCTGGTAACGGATAAGCACGTGGAGGGTTGGGA TGATCCCCGCATGCCTACTATTTCTGGGTTGCGTCGTCGCGGTTATACGGCTGCGTCGATCCGTGAGTTCTGTAAGCGCATCGGAGTGACCAAGCAAGACAACACCATTGAAATGGCCAGC CTTGAATCCTGCATTCGCGAGGACTTAAATGAAAATGCGCCACGCGCAATGGCCGTTATTGACCCCGTAAAGCTTGTGATCGAAAACTACCAGGGGGAGGGCGAGATGGTAACTATGCCAA ACCATCCTAACAAGCCGGAGATGGGAAGTCGTCAAGTCCCGTTTTCCGGAGAAATTTGGATTGATCGTGCCGATTTCCGCGAAGAGGCTAATAAACAATACAAGCGTTTGGTTCTTGGGAAG GAGGTACGTTTACGTAACGCATACGTAATTAAGGCCGAGCGCGTAGAAAAAGATGCGGAGGGTAACATCACCACGATCTTCGTCAAAAGACCCAGCCG ATGGCCGTAAGGTAAAGGGCGTAATCCATTGGGTGTCTGCTGCGCATGCGTTGCCGGTAGAAATTCGCTTGTACGACCGTTTGTTTTCAGTTCCGAACCCCGGTGCCGCAGATGATTTCTT GTCCGTTATTAATCCGGAATCTCTTGTTATTAAGCAAGGGTTCGCGGAGCCGTCTCTGAAAGACGCAGTTGCGGGTAAGGCTTTTCAATTCGAACGCGAGGGCTACTTCTGCTTGGACTCGC GCCACTCCACCGCAGAAAAGCCCGTTTTTAACCGCACCGTCGGCTTACGTGACACTTGGGCCAAAGTGGGAGAATAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGA AAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
GluRS	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCACCATGGTCACCATCACGGTGGTAGCGGTGGTGGTAGCGGTAA GATCAAGACCCGTTTCGCTCCGAGTCCAACGGGATACCTGCATGTCGGCGGCGCACGTACCGCATTGTATAGCTGGTTATTCGCGCGTAACCATGGAGGAGAATTTGTACTGCGTATTGAA GATACGGACCTTGAGCGTTCCACCCCGGAGGCTATCGAGGCTATTATGGATGGGATGAACTGGCTTAGTTTAGAATGGGACGAGGGACCCTATTATCAGACGAAGCGCTTCGATCGCTATA ACGCTGTGATTGATCAAATGCTTGAAGAAGGAACCGCTTACAAATGCTACTGCTCTAAGGAGCGCTTAGAAGCATTGCGCGAAGAGCAAATGGCTAAAGGAGAGAAACCCCGCTACGACGG TCGTTGTCGCCATTCCCATGAGCATCACGCAGACGACGAGCCGTGCGTCGTACGCTTCGCAAACCCGCAGGAAGGATCTGTTGTATTTGATGATCAGATTCGCGGGCCGATCGAGTTCTCA AACCAGGAGCTTGATGATTTGATTATCCGCCGTACCGACGGCTCGCCCACGTATAATTTTTGTGTCGTAGTTGACGATTGGGACATGGAAATCACCCACGTCATTCGCGGCGAAGACCACAT TAATAACACACCTCGCCAAATTAATATTCTTAAGGCGTTGAAAGCACCGGTTCCTGTTTACGCTCATGTTTCTATGATTAACGGGGATGACGGGAAAAAACTGTCGAAACGCCATGGTGCAGT	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)

	CTCTGTTATGCAGTACCGTGATGATGGGTACCTGCCAGAAGCGTTGCTGAACTACTTGGTGCGCCTTGGTTGGTCGCATGGAGATCAGGAAATTTTTACTCGTGAAGAGATGATCAAGTACT TTACTCTGAATGCTGTAAGCAAGAGTGCGAGCGCCTTCAACACGGACAAGTTGCTTTGGCTGAACCACCACTATATTAATGCGCTGCCACCTGAATACGTGGCTACGCACTTACAATGGCAT ATCGAGCAGGAGAACATTGATACGCGCAATGGTCCCCAGTTAGCGGACCTGGTAAAGCTGCTTGGAGAGCGTTGTAAGACTTTAAAAGAAATGGCACAAAGTTGCCGTTACTTTTATGAGGA CTTCGCTGAGTTCGATGCAGACGCTGCAAAGAAGCATCTGCGCCCGGTCGCACGCCAACCATTGGAAGTGGTTCGTGATAAGCTTGCAGCGATTACTGATTGGACGGCTGAAAATGTTCAT CACGCAATTCAGGCTACGGCCGATGAACTTGAAGTAGGAATGGGCAAAGTAGGTATGCCCCTTCGTGTTGCAGTCACAGGCGCTGGGCAGTCACCAGCATTAGACGTGACCGTACATGCC ATTGGTAAAACTCGCAGCATTGAGCGCATTAACAAGGCATTGGATTTCATTGCTGAGCGCGAAAATCAGCAGTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAG ACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'				
GlyRSα	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCAGAAGTTCGATACTCGTACTTTTCAGGGATTGATCTTGACTTTGCAAG ACTACTGGGCACGCCAAGGCTGCACAATCGTTCAGCCCTTAGATATGGAGGTCGGGGCCGGTACAAGTCACCCAATGACTTGTCTGCGCGAGCTTGGACCTGAACCTATGGCGGCAGCTT ACGTTCAGCCCTCACGCCGCCCCACGGACGGGCGCTACGGCGAAAATCCTAACCGCTTGCAACATTATTACCAGTTCCAAGTTGTAATCAAGCCATCCCCGGACAACATCCAAGAACTGTA TTTGGGTAGTCTGAAGGAATTGGGCATGGATCCCACCATTCACGATATTCGTTTCGTAGAGGACAATTGGGAGAACCCGACGTTAGGCGCGTGGGGGCTTGGTTGGGAGGTCTGGTTGAAC GGGATGGAGGTAACCCAGTTCACGTATTTTCAACAAGTTGGTGGTTTGGAATGTAAGCCTGTAACGGGCGAAATTACATATGGATTGGAGCGCCTGGCCATGTACATCCAGGGAGTCGATT CGGTGTATGATCTGGTATGGAGTGATGGACCCTTGGGAAAAACTACTTATGGCGATGTGTTTCATCAAAATGAGGTAGAACAATCAACTTATAATTTTGAGTATGCAGATGTGGATTTCTTATT TACTTGTTTCGAGCAGTATGAGAAAGAGGCGCAGCAATTGCTTGCCTTGGAAAACCCTTTGCCGTTGCCTGCGTATGAGCGTATCTTGAAGGCGGCTCACTCTTTCAATCTTCTTGATGCGC GTAAAGCTATCTCAGTCACCGAACGCCAACGTTACATTCTTCGCATTCGTACTTTAACTAAAGCGGTCGCAGAAGCCTACTACGCCAGTCGTGAGGCTTTAGGATTCCCAATGTGTAATAAGG ACAAGGGTGGTAGCGGTGGTGGTAGCGGTCATCATCATCATCATCATTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTG TTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pUC57	Kan	*E. coli* BL21-Gold (DE3)
GlyRSβ	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGAGTGAAAAGACATTCCTGGTAGAAATTGGCACTGAGGAGTTACCTCCT AAAGCTCTTCGTTCGCTTGCAGAATCCTTTGCCGCTAATTTCACGGCCGAATTGGATAATGCAGGCTTGGCCCATGGGACCGTACAGTGGTTCGCGGCCCCGCGCCGCCTTGCCCTTAAGG TCGCCAATTTAGCAGAGGCGCAGCCAGATCGTGAGATTGAGAAGCGCGGTCCAGCCATTGCACAAGCGTTCGACGCCGAGGGAAAGCCTTCGAAGGCAGCCGAAGGCTGGGCGCGTGGT TGTGGAATTACAGTCGACCAGGCTGAGCGTCTTACGACAGACAAAGGGGAATGGTTGTTATATCGCGCTCATGTAAAAGGCGAAGTCGCTTCCTAACATGGTGGCTACTT CGCTTGCGAAGTTGCCCATCCCGAAACTGATGCGTTGGGGCGCGTCAGACGTGCACTTTGTACGCCCTGTGCACACTGTTACCTTACTTTTGGGCGATAAAGTTATCCCTGCCACTATTTTA GGCATTCAGAGTGATCGTGTAATCCGCGGACATCGCTTCATGGGGGAGCCCGAGTTCACTATCGACAACGCAGACCAATATCCCGAAATTCTGCGCGAGCGTGGGAAGGTAATCGCTGAC TATGAAGAACGCAAAGCTAAGATTAAAGCGGACGCTGAGGAAGCTGCCCGCAAGATCGGAGGTAACGCCGACTTATCCGAATCTCTGCTGGAGGAAGTAGCGTCGTTAGTCGAGTGGCCC GTAGTGTTAACGGCAAAATTCGAGGAAAAATTCCTGGCTGTGCCTGCGGAAGCGCTGGTCTACACCATGAAAGGAGATCAAAAATATTTCCCCGTGTACGCTAATGATGGGAAGTTACTGCC GAATTTCATCTTTGTTGCGAATATCGAATCGAAAGACCCACAGCAAATTATTTCCGGCAACGAAAAAGTTGTCCGTCCACGCTTGGCGGATGCGGAGTTTTTTTTCAACACGGATCGCAAAAA ACGTCTTGAAGATAACCTTCCTCGTCTGCAAACCGTTTTGTTCCAGCAGCAATTGGGGACACTGCGTGACAAAACTGACCGCATCCAGGCACTTGCTGGGTGGATCGCAGAACAGATTGGA GCTGACGTGAATCACGCCACGCGCGCCGGCTTGTTAAGCAAATGCGATCTGATGACAAATATGGTCTTTGAATTTACCGATACGCAGGGGGTGATGGGAATGCATTACGCACGCCATGATG GTGAGGCGGAAGACGTCGCTGTCGCGTTAAACGAACAGTATCAGCCGCGCTTCGCAGGAGATGATCTGCCAAGCAATCCAGTAGCGTGTGCACTTGCTATCGCCGACAAAATGGGATACGTT AGCAGGCATCTTTGGGATTGGTCAACATCCGAAGGGAGATAAAGATCCCTTCGCGCTGCGTCGTGCAGCACTTGGAGTGTTGCGTATCATCGTGGAAAAAAACTTAAATCTGGATCTGCAAA CGCTTACAGAAGAGGCGGTTCGTTTATACGGGGACAAATTGACTAACGCAAACGTCGTCGACGATGTTATCGACTTCATGCTGGGACGCTTCCGCGCTTGGTATCAGGACGAAGGTTATAC AGTAGACACAATCCAGGCAGTCTTGGCCCGCCGCCCAACTCGCCCCGCCGATTTTGACGCACGCATGAAGGCGGTCTCCCATTTTCGTACGTTAGACGCCGCTGCTGCTTTGGCTGCGGC GAATAAGCGCGTGAGTAATATTCTGGCAAAGTCAGACGAGGTACTTAGTGACCGTGTAAACGCCTCAACCCTTAAGGAACCTGAGGAAATTAAGTTAGCTATGCAAGTCGTCGTATTGCGCG ATAAGTTGGAACCCTACTTTACGGAGGGTCGCTACCAAGATGCTTTAGTCGAATTAGCGGAATTGCGCGAACCAGTGGATGCGTTCTTTGACAAGGTGATGGTGATGGTAGACGATAAAGAA CTTCGTATCAATCGCTTAACTATGTTGGAGAAATTACGCGAGCTTTTCCTGCGCGTAGCAGATATCTCTTTATTACAAGGTGGTAGCGGTGGTGGTAGCGGTCATCATCATCATCATCATTAA TAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTG GGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
HisRS	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGGCGAAAAACATCCAAGCGATCCGTGGAATGAACGACTATTTGCCAGG GGAGACAGCCATTTGGCAACGCATCGAGGGTACGCTGAAGAATGTGCTGGGGTCGTATGGGTACTCTGAGATTCGCTTGCCCATTGTGGAGCAAACGCCCTTGTTTAAGCGCGCAATCGG AGAAGTCACTGATGTCGTTGAAAAAGAAATGTACACATTTGAGGACCGTAACGGTGATTCCTTGACCTTGCGTTCCAGAAGGCACGGCGGGCTGCGTTCGCGCTGAATTGAACATGGTTTG TTGTACAATCAAGAACAGCGCCTGTGGTACATCGGACCTATGTTCCGCCATGAGCGTCCACAGAAGGGGCGCTATCGCCAATTTCATCAATTGGGGTGTGAAGTGTTCGGTTTGCAAGGAC CCGATATCGATGCCGAACTTATTATGTTAACCGCCCGTTGGTGGCGCGCGTTAGGAATCTCGGAGCACGTAACACTGGAGCTGAACTCGATCGGTTCCTTAGAGGCGCGTGCCAACTACCG TGACGCCCTTGTCGCCTTTCTGGAACAGCACAAAGAAAAGCTGGATGAAGATTGTAAACGTCGTATGTACACTAATCCTCTGCGTGTTTTAGACTCGAAGAACCCCGAGGTGCAGGCACTTT TGAACGACGCACCCGCCCTGGGCGACTACTTGGACGAGGAAAGTCGCGAACATTTCGCTGGCTTATGTAAGCTGTTGGAAAGTGCCGGAATTGCTTATACTGTGAATCAGCGCCTTGTTCG CGGCCTGGACTACTACAATCGTACTGTTTTTGAGTGGGTCACTAACTCACTGGGTAGCCAGGGTACTGTGTGCGCTGGGGGTCGCTACGACGGTTTAGTTGAGCAACTGGGGGGGCGTGC GACCCCCGCTGTTGGATTTGCCATGGGATTAGAACGTTTGGTGCTTTTGGTGCAAGCTGTCAACCCCGAGTTCAAGGCCGACCCCGTAGTTGACATTTACCTTGTGGCAAGTGGAGCAGAT ACGCAGTCAGCCGCCATGGCATTAGCTGAACGTCTGCGCGACGAGTTACCCGGTGTCAAATTGATGACAAACCACGGTGGGGGGAACTTTAAGAAACAATTTGCCCGTGCCGACAAATGG GGAGCACGCGTCGCCGTTGTACTGGGGGAGTCTGAAGTAGCCAATGGGACTGCTGTCGTGAAGGACCTGCGTTCAGGAGAACAAACTGCGGTGGCCCAGGATAGTGTAGCAGCCCATTTA CGCACACTTTTGGGAGGTGGTAGCGGTGGTGGTAGCGGTCATCATCATCATCATCATTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTT TTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pJET	Amp	*E. coli* BL21-Gold (DE3)
IleRS	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCATCATCATCATCATGGTGGTAGCGGTGGTGGTAGCGGTAGCGA TTATAAATCCACCCTTAATTTGCCAGAGACGGGATTCCCTATGCGTGGAGACCTGGCAAAGCGCGAGCCGGGGATGCTGGCACGTTGGACCGACGATGACCTGTACGGCATCATTCGTGC GGCTAAAAAGGGTAAAAAGACTTTCATCTTACATGATGGCCCTCCCTACGCGAATGGCAGTATTCACATTGGACACAGCGTGAACAAGATTTTGAAGGATATCATCGTAAAGTCCAAGGGCTT ATCCGGGTACGATTCTCCTTATGTTCCGGGGTGGGATTGCCACGGCCTTCCTATCGAATTGAAAGTGGAACAAGAATACGGTAAGCCGGGAGAAAAATTTACCGCCGCTGAGTTCCGTGCT AAGTGTCGTGAGTACGCAGCGACTCAGGTAGACGGCCAGCGCAAGGACTTCATCCGTCTTGGGGTCCTGGGAGACTGGAGCCATCCATATCTGACTATGGATTTCAAAACTGAAGCGAATA TCATCCGCGCGCTGGGCAAAATCATTGGTAACGGGCACCTGCACAAAGGGGCTAAACCGGTTCACTGGTGCGTCGATTGTCGTAGTGCCTTAGCAGAGGCTGAGGTGGAATATTACGACA AGACCTCCCCGTCGATTGACGTTGCATTTCAAGCAGTGGATCAGGACGCTCTGAAAGCGAAATTTGCGGTCAGCAATGTGAACGGCCCCATTTCTTTAGTTATCTGGACCACAACACCTTGG	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)

	ACCTTACCGGCTAATCGCGCTATCAGCATTGCCCCAGATTTTGATTACGCGCTGGTCCAGATCGATGGGCAGGCGGTGATCTTAGCGAAGGACCTGGTGGAGTCGGTAATGCAACGCATTG GCGTCACCGACTACACCATCCTGGGTACGGTAAAGGGTGCGGAGCTTGAATTGTTGCGTTTCACGCATCCGTTTATGGGTTTCGATGTTCCCGCCATCTTAGGCGATCATGTTACTCTTGAT GCAGGCACAGGCGCTGTCCATACCGCCCCTGGTCATGGTCCTGACGATTACGTGATTGGACAGAAATACGGTTTGGAAACCGCTAATCCAGTGGGGCCCGACGGTACCTACCTGCCTGGA ACGTACCCAACATTAGATGGTGTAAACGTATTTAAAGCGAACGACATTGTTGTTGCACTTTTGCAGGAGAAGGGTGCCTTATTGCATGTCGTGTTGCACTGTTGCTGG CGCCATAAGACCCCCATCATCTTCCGTGCTACCCCACAATGGTTCGTGTCTATGGATCAAAAGGGGCTTCGCGCTCAATCATTGAAAGAGATTAAGGGCGTTCAGTGGATCCCCGATTGGG GACAAGCCCGTATTGAGAGCATGGTCGCCAATCGCCCCGACTGGTGCATCTCGCGCCAGCGCACCTGGGGGGTCCCAATGTCGTTGTTTGTTCACAAAGATACGGAAGAGCTGCACCCCC GTACTCTGGAACTGATGGAGGAGGTCGCGAAACGTGTCGAAGTTGATGGGATTCAAGCTTGGTGGGATTTAGACGCTAAAGAAATTTTAGGGGACGAAGCCGACCAATATGTCAAGGTACC CGACACGTTGGATGTCTGGTTTGATTCTGGTAGCACCCATTCATCCGTTGTCGATGTGCGTCCGGAATTTGCCGGACATGCCGCCGATATGTATTTGGAAGGGAGTGACCAACATCGCGGG TGGTTTATGTCCTCTTTAATGATCTCTACGGCCATGAAGGGCAAGGCCCCGTATCGCCAGGTATTGACTCATGGCTTCACAGTGGACGGTCAAGGCCGTAAAATGTCTAAGAGTATCGGTAA CACGGTCAGCCCGCAAGACGTAATGAACAAATTAGGCGCGGACATTTTGCGCCTTTGGGTTGCGAGCACGGATTATACTGGTGAGATGGCAGTTAGTGACGAGATTCTCAAGCGCGCGGC AGACTCCTACCGCCGTATCCGTAACACCGCGCGTTTCTTGCTTGCGAACTTAAATGGGTTTGACCCTGCCAAGGACATGGTGAAACCTGAGGAAATGGTCGTCTTAGATCGCTGGGCTGTT GGGTGTGCAAAGGCTGCACAGGAAGACATTTTAAAGGCATACGAAGCATACGATTTTCACGAGGTCGTGCAGCGTCTTATGCGCTTCTGCTCCGTGGAGATGGGTAGTTTCTATTTGGATAT CATTAAAGATCGCCAGTATACAGCCAAGGCCGATAGCGTGGCACGCCGTTCGTGTCAAACTGCGTTGTACCATATTGCGGAGGCCTTAGTGCGCTGGATGGCGCCAATTTTGTCGTTCACT GCCGACGAGGTCTGGGGCTACCTGCCGGGAGAACGTGAGAAGTACGTCTTCACAGGGGAATGGTATGAGGGACTGTTCGGCCTTGCTGATAGCGAGGCGATGAACGACGCATTCTGGGA TGAACTTTTAAAGGTCCGTGGGGAAGTCAATAAGGTCATTGAGCAGGCTCGTGCGGACAAGAAAGTAGGCGGGAGTTTGGAGGCCGCAGTGACACTTTATGCTGAGCCTGAATTAAGCGCA AAACTTACGGCTTTGGGTGACGAACTTCGCTTCGTCTTACTTACCTCAGGGGCCACCGTAGCTGATTACAATGACGCACCTGCCGATGCTCAACAGTCCGAGGTCCTGAAGGGCTTAAAAGT GGCCTTGTCGAAAGCCGAAGGTGAGAAATGTCCACGCTGCTGGCACTATACTCAGGACGTAGGCAAAGTGGCCGAGCACGCAGAAATTTGTGGACGCTGTGTAAGTAATGTTGCCGGAGA TGGCGAGAAGCGCAAATTTGCTTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGA GTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3’				
LeuRS	5’- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCATCATCATCATCATGGTGGTAGCGGTGGTGGTAGCGGTCAGGA GCAGTATCGTCCAGAAGAGATTGAGAGCAAGGTCCAATTACATTGGGACGAAAAGCGCACATTTGAGGTTACAGAAGATGAGTCCAAAGAAAAGTACTACTGTTTATCAATGCTGCCCTACC CATCTGGGCGTTTGCATATGGGTCATGTTCGTAATTATACTATTGGGGACGTGATTGCGCGCTATCAGCGTATGCTTGGAAAAAATGTCTTACAACCGATTGGCTGGGACGCATTTGGCTTG CCGGCAGAAGGTGCGGCGGTGAAGAATAATACGGCACCAGCCCCATGGACATACGACAACATTGCATACATGAAGAATCAATTAAAGATGCTGGGGTTTGGATATGATTGGTCTCGTGAGT TAGCAACGTGTACACCGGAATACTACCGTTGGGAGCAGAAGTTTTTCACAGAGTTATATAAAAAGGGATTAGTCTATAAAAAAACGTCGGCGGTGAACTGGTGTCCAAACGACCAGACTGTT CTGGCTAACGAACAGGTCATCGATGGTTGCTGCTGGCGCTGCGACACAAAAGTTGAACGTAAAGAAATTCCACAATGGTTTATCAAGATTACCGCATACGCTGATGAGTTGCTTAACGACCT TGACAAACTTGACCACTGGCCAGATACTGTTAAAACTATGCAGCGTAACTGGATCGGACGCTCCGAAGGGGTGGAGATCACTTTTAATGTTAACGACTACGACAACACGTTGACAGTATATA CTACTCGCCCGGACACATTCATGGGATGTACCTATCTTGCAGTAGCAGCGGGACATCCGCTGGCCCAGAAAGCGGCAGAAAACAATCCGGAGTTAGCTGCCTTCATTGATGAGTGCCGTAA CACTAAGGTGGCGGAAGCTGAGATGGCTACCATGGAGAAGAAGGGCGTGGATACAGGCTTCAAGGCCGTCCACCCCCTGACGGGGGAGGAGATTCCGGTTTGGGCCGCTAACTTTGTGT TGATGGAATACGGGACTGGAGCGGTCATGGCCGTACCCGGCCATGATCAGCGCGATTATGAGTTTGCTTCAAAATACGGGTTAAATATCAAACCCGTGATCTTGGCCGCAGACGGATCAGA ACCGGACTTGTCGCAACAGGCGTTAACTGAGAAGGGCGTCCTGTTTAATAGTGGAGAGTTCAACGGCCTTGATCATGAGGCTGCATTCAATGCTATTGCAGATAAGTTGACGGCGATGGGA GTAGGCGAACGTAAGGTCAACTACCGCCTTCGCGACTGGGGAGTATCCCGCCAGCGTTACTGGGGTGCACCAATTCCTATGGTTACGTTGGAAGATGGCACGGTAATGCCCACGCCTGAC GACCAGCTGCCCGTCATCCTTCCCGAAGATGTCGTAATGGACGGCATTACGAGTCCAATTAAAGCTGATCCCGAGTGGGCAAAAACAACTGTGAATGGCATGCCGGCCTTACGTGAAACCG ACACGTTTGACACCTTTATGGAGTCGAGTTGGTATTACGCCCGTTACACTTGCCCTCAGTATAAGGAAGGGATGCTTGACTCGGAAGCAGCTAATTACTGGTTGCCAGTAGACATCTATATTG GCGGCATTGAGCATGCGATCATGCATCTTCTGTATTTCCGTTTTTTTCATAAACTGATGCGTGATGCAGGAATGGTCAACAGTGATGAGCCCGCCAAACAACTGTTGTGCCAGGGTATGGTG CTGGCTGACGCTTTTTACTACGTCGGTGAAAATGGAGAGCGCAACTGGGTTTCACCAGTAGATGCGATTGTAGAGCGCGATGAGAAAGGCCGCATCGTTAAGGCTAAAGACGCTGCCGGG CACGAGCTTGTTTATACTGGAATGAGCAAAATGAGTAAGTCCAAGAATAACGGGATCGACCCACAAGTGATGGTGGAACGCTACGGTGCTGACACGGTGCGTCTGTTTATGATGTTCGCGT CTCCAGCAGATATGACTCTTGAGTGGCAGGAGTCTGGGGTCGAAGGAGCTAATCGCTTTTTGAAGCGCGTGTGGAAATTAGTATACGAGCACACGGCGAAGGGCGATGTGGCTGCTTTGAA CGTCGATGCCCTGACCGAAAATCAAAAAGCCCTTCGTCGTGACGTGCATAAGACCATTGCGAAGGTAACCGACGATATCGGCCGTCGTCAAACATTCAATACAGCTATTGCTGCGATTATGG AGCTGATGAACAAGTTGGCCAAAGCTCCCACTGACGGGGAACAGGACCGCGCACTTATGCAGGAAGCTCTTTTGGCGGTGGTACGTATGTTAAATCCCTTTACGCCCCACATCTGTTTCAC CTTGTGGCAAGAACTGAAGGGTGAGGGCGATATTGATAATGCACCCTGGCCTGTCGCAGACGAGAAGGCCATGGTGGAAGACTCCACGCTGGTGGTAGTGCAGGTCAACGGGAAGGTTC GCGCAAAAATTACCGTGCCTGTGGACGCCACTGAAGAACAGGTACGTGAGCGCGCAGGGCAGGAACATCTGGTGGCCAAATACCTGGACGGTGTGACGGTGCGTAAAGTTATCTATGTGC CAGGCAAGTTACTTAATTTGGTAGTGGGTTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTC TACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3’	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
LysRS	5’- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCATCATCATCATCATGGTGGTAGCGGTGGTGGTAGCGGTAGCGA ACAACATGCTCAAGGAGCTGACGCCGTCGTGGACCTGAACAACGAGTTAAAAACTCGTCGTGAAAAACTTGCAAATCTGCGTGAGCAGGGGATCGCATTCCCGAACGACTTTCGTCGTGAC CACACTTCTGACCAACTTCATGCTGAGTTTGATGGTAAAGAAAATGAAGAGTTAGAAGCATTAAACATCGAGGTTGCCGTTGCGGGGCGCATGATGACACGTCGCATCATGGGCAAGGCATC CTTCGTCACCTTACAGGATGTTGGTGGGCGTATTCAACTGTACGTCGCACGTGATGATTTGCCCGAGGGTGTTTACAACGAACAGTTTAAGAAATGGGATCTTGAGATATCTTAGGGGCGA AGGGGAAGTTATTCAAGACAAAAACTGGCGAGCTTTCGATTCACTGTACCGAGTTACGCTTACTGACCAAGGCATTACGCCCTCTTCCGGACAAATTCCATGGGCTGCAAGACCAAGAAGCC CGTTACCGCCAGCGTTATTTAGACCTTATTTCGAATGACGAGTCTCGTAACACATTTAAAGTCCGTTCGCAGATTCTGTCTGGCATCCGTCAGTTTATGGTCAATCGCGGCTTCATGGAGGTA GAGACCCCGATGATGCAAGTCATCCCTGGCGGCGCGGCAGCTCGCCCTTTCATCACTCACCATAATGCGCTTGATTTGGATATGTATTTGCGCATTGCACCTGAGTTGTATCTTAAACGCCT GGTTGTCGGAGGCTTTGAGCGTGTCTTTGAAATTAACCGCAATTTCCGTAACGAGGGAATCAGTGTCCGTCACAATCCAGAGTTCACTATGATGGAGCTGTACATGGCCTATGCAGACTATA AAGACTTAATTGAACTTACCGAGTCATTGTTCCGCACGTTGGCCCAAGACATCTTAGGAAAAACTGAGGTAACGTATGGCGACGTAACCTTAGACTTCGGAAAACCGTTTGAAAAGCTGACG ATGCGTGAAGCGATCAAAAAGTATCGCCCAGAAACGGATATGGCAGACTTAGACAATTTCGACTCTGCGAAAGCCATCGCAGAATCAATTGGTATTCATGTGGAGAAATCCTGGGGGCTTGG TCGTATCGTCACAGAAATTTTTGAGGAGGTAGCAGAGGCCCACCTTATTCAACCCACATTCATCACTGAGTATCCGGCGGAAGTCTCACCATTGGCTCGTCGCAATGATGTCAACCCCGAGA TCACGGACCGCTTCGAGTTCTTTATTGGGGGCCGTGAAATTGGGAATGGGTTTTCTGAACTTAACGATGCTGAAGACCAAGCGCAACGTTTCTTGGACCAGGTGGCCGCTAAAGACGCCGG CGACGACGAGGCAATGTTCTACGATGAGGATTATGTTACTGCACTGGAGCACGGCCTTCCCCCCACTGCCGGCTTAGGGATCGGCATCGACCGCATGGTTATGCTGTTCACAAACTCGCAC ACCATCCGTGACGTTATTTTATTCCCCGCCATGCGCCCAGTCAAAATAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTT TGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3’	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)

MetRS	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGACACAGGTAGCAAAGAAAATTTTAGTTACCTGTGCTTTACCATATGCGA ATGGATCAATCCACCTTGGCCACATGCTGGAGCATATCCAGGCTGACGTTTGGGTGCGCTATCAACGTATGCGCGGTCACGAGGTCAACTTTATCTGCGCGGATGACGCGCATGGCACACC GATCATGCTTAAAGCGCAACAGTTAGGTATTACGCCAGAACAAATGATCGGCGAGATGAGCCAAGAACATCAAACCGATTTCGCTGGCTTTAACATCAGCTATGACAACTACCATTCGACAC ATTCGGAAGAAAATCGCCAATTGTCAGAGCTGATCTATTCCCGCTTGAAAGAAAATGGGTTCATTAAAAATCGTACTATTAGCCAGTTGTACGACCCTGAAAAGGGTATGTTTTTGCCGGATC GTTTTGTTAAAGGGACTTGCCCCAAGTGCAAGTCACCTGACCAGTACGGTGATAACTGTGAGGTCTGTGGGGCGACCTACTCGCCCACTGAGTTAATCGAGCCGAAATCTGTCGTGTCCGG CGCGACCCCTGTGATGCGCGATAGTGAACACTTCTTTTTTGATTTACCTAGTTTTTCTGAAATGCTTCAGGCCTGGACTCGTTCCGGGGCGTTGCAGGAACAGGTCGCCAATAAGATGCAAG AATGGTTCGAGAGCGGGTTACAGCAATGGGATATCTCACGTGATGCCCCATACTTCGGGTTTGAGATCCCTAACGCACCGGGGAAGTACTTCTATGTTTGGCTTGATGCCCCTATTGGATAT ATGGGCAGCTTCAAAAATTTATGCGATAAGCGTGGAGATTCAGTCTCCTTTGACGAATACTGGAAAAAGGACAGCACCGCAGAACTTTACCATTTCATTGGAAAGGATATCGTTTACTTCCAC AGTTTGTTTTGGCCTGCCATGCTGGAAGGCAGTAATTTCCGTAAACCTTCAAATCTTTTTGTTCATGGTTATGTGACGGTTAATGGTGCCAAGATGTCCAAAAGCCGTGGAACTTTCATCAAA GCGTCAACATGGTTGAATCACTTCGACGCCGATTCCCTGCGCTATTATTACACCGCTAAGCTTTCTTCGCGCATCGACGACATCGACCTTAATCTGGAAGATTTTGTACAGCGTGTTAATGCC GACATTGTGAACAAAGTAGTCAATTTGGCTTCACGTAACGCGGGTTTTATCAACAAGCGTTTTGACGGCGTCTTAGCTTCTGAATTAGCAGACCCCCAGTTATACAAAACCTTCACTGATGCG GCTGAAGTAATTGGTGAAGCGTGGGAGTCTCGTGAGTTCGGCAAAGCCGTTCGTGAAATTATGGCCCTTGCTGACTTGGCAAACCGCTATGTAGACGAGCAAGCACCCTGGGTGGTGGCT AAGCAAGAGGGGCGCGATGCCGATCTTCAAGCAATCTGCTCTATGGGAATTAACCTTTTCCGCGTATTGATGACATATTTGAAGCCTGTGTTACCTAAGTTAACTGAGCGCGCTGAGGCGTT TTTAAACACGGAACTGACCTGGGATGGCATCCAACAGCCTTTGTTGGGGCATAAAGTTAATCCATTCAAGGCATTATACAATCGCATCGATATGCGCCAAGTTGAGGCCTTAGTAGAGGCTT CTAAGGAAGAAGTTAAAGCCGCTGCGGCTCCCGTCACCGGACCATTGGCTGACGATCCGATTCAAGAAACAATCACTTTCGATGACTTTGCAAAGGTCGACCTTCGTGTTGCCCTGATTGAG AACGCGGAGTTTGTCGAAGGCTCTGATAAGTTGCTTCGCTTGACACTGGATCTTGGGGGAGAAAAACGTAACGTTTTTAGTGGAATCCGCAGTGCATACCCTGATCCACAGGCACTTATTGG TCGCCATACGATCATGGTGGCGAATCTTGCGCCTCGCAAGATGCGCTTTGGAATCTCAGAGGGAATGGTGATGGCGGCTGGGCCTGGTGGAAAGGACATCTTTCTGTTGAGTCCTGACGC TGGAGCTAAGCCGGGCCATCAGGTGAAAGGTGGTAGCGGTGGTGGTAGCGGTCATCATCATCATCATTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGA CTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
PheRS**α**	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCATCATCATCATCATGGTAGCGGTGGTGGTAGCGGTGGTAGCCA CCTTGCTGAATTGGTAGCAAGTGCAAAGGCGGCTATCTCCCAAGCTTCTGACGTAGCAGCGCTGGACAATGTTCGCGTGGAGTATCTGGGAAAGAAAGGGCACTTAACATTGCAAATGACA ACACTTCGTGAGTTGCCCCCCGAGGAACGTCCCGCCGCAGGTGCAGTGATTAACGAAGCTAAGGAACAAGTGCAGCAAGCACTTAACGCGCGCAAAGCAGAGTTAGAATCCGCGGCACTG AACGCGCGTTTAGCTGCCGAAACTATTGATGTTAGTCTGCCTGGCCGCCGCATCGAAAATGGTGGGTTACATCCAGTCACTCGTACCATTGATCGCATTGAATCTTTCTTCGGCGAAGCTGGG TTTCACTGTTGCAACTGGCCCTGAGATCGAAGATGACTACCACAACTTCGATGCACTGAATATCCCAGGCCACCATCCTGCGCGCGCCGACCATGATACCTTCTGGTTTGACACTACCCGTC TGTTACGCACCCAAACGAGTGGTGTCCAAATTCGCACCATGAAGGCCCAACAGCCACCGATCCGTATTATTGCTCCGGGGCGCGTTTACCGTAATGACTACGACCAGACGCACACACCAAT GTTCCACCAAATGGAGGGGCTTATCGTAGACACGAATATCAGTTTTACCAACCTGAAGGGAACGCTTCACGATTTTCTTCGTAATTTCTTTGAGGAGGATTTGCAGATTCGTTTCCGCCCGTC CTATTTTCCGTTTACAGAGCCCTCGGCGGAAGTCGATGTAATGGGGAAAAATGGGAAGTGGCTGGAAGTCCTGGGGTGTGGTATGGTGCACCCAAATGTCCTTCGCAATGTTGGAATTGAC CCAGAAGTCTATTCCGGATTTGCCTTTGGAATGGGAATGGAGCGTCTGACTATGTTACGCTACGGTGTGACGGACCTTCGCTCCTTTTTTGAGAATGATCTTCGTTTCTTGAAGCAGTTTAAA TAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGG TGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
PheRS**β**	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGAAGTTTTCTGAACTGTGGCTTCGCGAGTGGGTCAACCCCGCAATCGA CAGCGATGCCCTGGCTAATCAGATTACCATGGCAGGTTTGGAGGTCGATGGAGTAGAGCCTGTGGCCGGTTCCTTTCATGGCGTTGTGGTGGGAGAGGTGGTCGAGTGTGCCCAACACCC GAATGCCGACAAACTTCGCGTTACGAAGGTCAATGTAGGGGGCGACCGTCTTTTAGACATTGTCTGCGGTGCGCCTAATTGCCGTCAAGGCTTGCGCGTGGCAGTAGCAACGATTGGCGC TGTATTGCCAGGTGACTTCAAAATCAAGGCGGCAAAGTTGCGCGGAGAACCGTCCGAAGGCATGTTATGTAGTTTCAGTGAGTTAGGTATTTCGGACGATCACAGTGGGATTATCGAGTTGC CAGCCGATGCCCCTATCGGTACAGATATTCGCGAGTATCTTAAGTTAGACGATAATACAATCGAGATTTCGGTAACACCTAATCGTGCAGATTGCTTAGGTATTATCGGCGTTGCGCGTGATG TAGCGGTGCTGAATCAATTACCGCTGGTGCAGCCTGAGATCGTACCCGTGGGTGCAACTATTGATGATACGCTGCCGATCACTGTGGAAGCACCCGAGGCTTGCCCACGCTATCTGGGTC GTGTCGTTAAGGGAATCAATGTCAAAGCACCGACACCATTATGGATGAAGGAGAAGTTGCGTCGTTGTGGCATCCGCTCTATCGACGCCGTCGTAGATGTTACTAATTACGTATTGCTGGAA CTGGGCCAGCCGATGCATGCGTTTGATAAGGACCGCATCGAAGGCGGGATTGTAGTTCGCATGGCTAAAGAAGGCGAGACGTTGGTACTGCTTGATGGAACGGAGGCAAAGCTTAATGCG GACACATTAGTCATCGCAGACCACAATAAGGCGCTTGCGATGGGCGGCATCTTTGGGGGCGAGCACAGCGGTGTAAACGACGAAACTCAGAACGTCCTGCTGGAGTGCGCATTCTTTTCTC CCTTAAGCATTACAGGGCGCGCACGCCGTCACGGTCTTCACACGGATGCATCCCACCGTTACGAGCGCGGCGTCGACCCCGCGTTACAACATAAAGCGATGGAACGCGCCACCCGCCTGT TAATCGACATTTGTGGGGGAGAAGCTGGACCAGTAATTGACATCACCAACGAGGCCACGCTGCCCAAACGCGCGACGATTACATTGCGTCGTTCTAAATTGGATCGCCTTATTGGACACCA CATCGCCGACGAACAAGTTACTGACATTCTGCGTCGTCTGGGCTGCGAGGTTACCGAAGGGAAGGACGAGTGGCAAGCCGTTGCCCCATCGTGGCGCTTTGATATGGAGATTGAAGAAGA TTTGGTTGAAGAGGTAGCTCGCGTCTACGGTTACAATAACATTCCGGACGAACCCGTGCAGGCTTCGCTTATCATGGGTACGCACCGTGAGGCCGATTTGTCGCTGAAGCGCGTTAAGACC CTTCTTAACGATAAGGGTTATCAGGAAGTTATTACGTATAGCTTTGTTGATCCAAAGGTCCAACAAATGATTCATCCAGGCGTAGAGGCCCTTCTGCTGCCGTCACCCATCTCTGTGGAGATG TCCGCCATGCGCCTGTCCTTGTGGACTGGCTTGTTGGCCACGGTTGTATATAACCAGAACCGCCAACAAAATCGCGTTCGTATCTTTGAGAGTGGTCTGCGCTTCGTGCCAGACACCCAAG CCCCCCTGGGCATTCGTCAAGACCTGATGTTGGCAGGGGTTATTTGCGGAAATCGTTACGAGGAACACTGGAATCTGGCCAAGGAGACGGTTGATTTCTACGACCTGAAAGGAGATCTTGA ATCGGTCTTAGACCTGACGGGGAAATTAAACGAGGTAGAGTTCCGCGCCGAAGCAAACCCGGCTCTTCACCCAGGTCAATCAGCAGCGATTTATTTAAAAGGTGAGCGTATTGGTTTCGTC GGTGTAGTTCACCCAGAATTGGAGCGTAAGTTAGACTTAAATGGACGCACATTGGTTTTTGAATTAGAGTGGAACAAACTTGCTGACCGTGTAGTACCGCAGGCTCGCGAAATCAGCCGCTT CCCTGCTAACCGTCGCGATATCGCAGTTGTCGTCGCTGAAAATGTGCCCGCGGCAGATATTTTGAGCGAGTGTAAAAAGGTAGGGGTCAATCAGGTCGTGGGTGTCAACTTGTTCGATGTT TATCGCGGTAAGGGCGTAGCTGAGGGATATAAATCGCTGGCAATTTCACTGATTTTACAGGATACTAGCCGCACTCTGGAGGAGGAAGAGATTGCAGCCACTGTGGCCAAGTGTGTAGAAG CGTTAAAAGAACGTTTCCAGGCCTCGCTGCGCGATGGTGGTAGCGGTGGTGGTAGCGGTCATCATCATCATCATTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTC GAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pUC57	Kan	*E. coli* BL21-Gold (DE3)
ProRS	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCGTACTTCCCAGTATCTTCTGTCGACTTTAAAAGAGACTCCTGCTGAT GCCGAGGTTATCAGCCACCAACTGATGTTACGTGCAGGGTATGATTCGCAAGCTGGCAAGTGGGGCTTTACACATGGTTACCCACGGGGGTGCGTGTACTGAAAAAGGTTGAGAACATTGTAC GTGAGGAAATGAACAACGCTGGTGCCATTGAAGTTTCCATGCCTGTCGTGCAACCAGCAGATTTATGGCAAGAATCTGGACGCTGGGAGCAATATGGCCCAGAGCTTTTGCGTTTCGTCGA CCGCGGGGAGCGTCCTTTCGTATTGGGCCCGACCCACGAAGAGGTTATTACGGATTTGATCCGCAACGAGTTAAGCTCATATAAACAGCTTCCACTTAACTTTTACCAAATCCAGACCAAGT TCCGTGATGAAGTCCGCCCACGTTTTGGAGTTATGCGTTCTCGCGAGTTCTTAATGAAAGACGCCTATTCGTTCCATACATCACAAGAGTCTTTACAGGAGACCTACGACGCAATGTACGCT	*E. coli*	pUC57	Kan	*E. coli* BL21-Gold (DE3)

	GCATATTCTAAGATTTTTTCGCGTATGGGATTAGATTTCCGCGCCGTTCAAGCAGACACTGGCTCTATCGGAGGGTCCGCGAGTCATGAATTTCAGGTATTAGCGCAGAGTGGGGAGGATGA CGTCGTTTTTTCAGATACGTCAGACTACGCTGCGAACATTGAGTTGGCCGAAGCAATTGCTCCAAAAGAACCGCGTGCAGCAGCCACCCAAGAAATGACGCTGGTCGATACGCCTAATGCC AAGACCATCGCAGAACTGGTCGAGCAGTTCAACCTGCCGATTGAGAAAACGGTGAAGACGCTTCTGGTTAAGGCTGTAGAGGGCTCCTCATTTCCCCAAGTAGCGTTGTTAGTTCGTGGGG ACCACGAGTTAAACGAGGTAAAAGCGGAAAAGCTGCCACAGGTCGCAAGTCCCCTTACGTTTGCCACAGAGGAGGAGATCCGCGCGGTTGTCAAAGCCGGACCAGGGAGCCTGGGACCC GTGAATATGCCAATTCCGGTGGTGATTGATCGCACTGTCGCCGCAATGTCGGATTTCGCAGCGGGAGCTAATATCGACGGAAAACACTACTTCGGTATTAATTGGGATCGCGATGTTGCAAC TCCTGAGGTAGCCGATATCCGCAACGTGGTCGCGGGAGATCCATCACCCGATGGGCAAGGTCGCCTGTTGATCAAGCGCGGAATCGAAGTGGGGCACATCTTCCAATTGGGCACGAAATA TTCTGAAGCTCTGAAGGCTAGCGTTCAGGGAGAAGATGGGCGCAACCAGATTCTGACAATGGGGTGCTACGGGATCGGTGTCGCTGCCGTCACCCGTGTCGTCGCTGCCGCCATCGAACAGAATTATGA CGAACGCGGGATCGTGTGGCCCGATGCCATTGCCCCGTTCCAAGTTGCAATTCTGCCTATGAACATGCATAAAAGCTTTCGCGTTCAAGAACTGGCTGAGAAACTTTACTCGGAACTTCGCG CACAGGGAATCGAAGTCCTGCTTGACGACCGTAAAGAACGCCCTGGCGTAATGTTCGCTGATATGGAGCTGATTGGAATCCCACACACTATTGTACTGGGTGATCGCAACCTTGATAACGAT GATATCGAGTACAAGTACCGTCGTAATGGAGAGAAGCAGTTGATTAAGACTGGCGATATTGTCGAATATTTGGTGAAACAAATCAAAGGGGGTGGTAGCGGTGGTGGTAGCGGTCATCATC ATCATCATCATTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGC TCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'				
SerRS	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGTTGGACCCAAATTTGTTACGCAACGAGCCAGACGCGGTCGCTGAGAA GCTTGCCCGCCGCGGCTTTAAGTTAGACGTTGATAAGTTAGGGGCACTTGAAGAGCGTCGTAAGGTTCTTCAAGTGAAGACCGAGAACTTACAAGCTGAACGCAATTCTCGCAGCAAATCG ATCGGTCAAGCGAAAGCACGTGGGGAGGACATCGAACCGCTTCGTTTAGAAGTAAATAAGTTAGGGGAAGAACTTGACGCCGCGAAAGCTGAGCTGGATGCCCATCAGGCAGAAATTCGT GACATTGCGTTAACGATTCCAAATTTGCCAGCAGATGAAGTCCCCGTGGGCAAAGATGAGAACGACAATGTGGAGGTCAGTCGTTGGGGAACACCGCGTGAGTTTGATTTCGAGGTGCGCG ACCACGTCACTCTGGGTGAAATGCACAGTGGCCTGGACTTCGCCGCGGCCGTAAAACTTACAGGCTCACGTTTCGTCGTAATGAAGGGGCAGATTGCCCGTATGCATCGCGCCCTTAGTCA GTTCATGTTAGACTTGCATACAGAACAACACGGGTATTCGGAGAATTATGTCCCCTACTTGGTTAATCAAGATACATTGTATGGGACAGGGCAGCTTCCCAAATTTGCTGGGGATCTGTTCCA CACTCGCCCTTTAGAGGAAGAAGCTGATACGAGTAACTACGCGCTTATCCCGACTGCGGAGGTGCCGTTGACTAATTTAGTACGCGGGGAAATTATCGACGAGGACGACTTACCCATCAAA ATGACAGCACATACTCCATGCTTTCGCTCGGAGGCAGGCAGTTACGGTCGTGACACTCGCGGTTTGATCCGTATGCACCAGTTTGATAAAGTTGAAATGGTCCAAATTGTTCGCCCCGAAGA TAGTATGGCTGCCTTGGAGGAAATGACCGGGCACGCGGAAAAGGTTCTTCAATTATTGGGCCTGCCCTACCGTAAGATCATTTTGTGTACAGGAGACATGGGCTTCGGTGCGTGCAAAACC TATGACCTGGAGGTATGGATTCCAGCACAGAATACATATCGCGAAATCTCATCCTGTTCTAACGTATGGGATTTTCAAGCTCGCCGCATGCAAGCACGTTGTCGCAGCAAGTCGGATAAGAA GACTCGCCTGGTTCACACGCTGAATGGGTCCGGCTTAGCAGTAGGCCGTACATTAGTGGCGGTGATGGAAAATTACCAACAAGCCGATGGCCGCATCGAAGTTCCAGAGGTTCTTCGCCCT TACATGAACGGCTTAGAATATATCGGGGGTGGTAGCGGTGGTGGTAGCGGTCATCATCATCATCATCATTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACT GGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
ThrRS	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCATCATCATCATCATGGTGGTAGCGGTGGTGGTAGCGGTCCGGT GATTACCCTGCCGGATGGCAGCCAGCGCCATTATGATCATGCGGTGAGCCCGATGGATGTGGCGCTGGATATTGGCCCGGGCCTGGCGAAAGCGTGCATTGCGGGCCGCGTGAACGGC GAACTGGTGGATGCGTGCGATCTGATTGAAAACGATGCGCAGCTGAGCATTATTACCGCGAAAGATGAAGAAGGCCTGGAAATTATTCGCCATAGCTGCGCGCATCTGCTGGGCCATGCGA TTAAACAGCTGTGGCCGCATACCAAAATGGCGATTGGCCCGGTGATTGATAACGGCTTTTATTATGATGTGGATCTGGATCGCACCCTGACCCAGGAAGATGTGGAAGCGCTGGAAAAACG CATGCATGAACTGGCGGAAAAAAACTATGATGTGATTAAAAAAAAAGTGAGCTGGCATGAAGCGCGCGAAACCTTTGCGAACCGCGGCGAAAGCTATAAAGTGAGCATTCTGGATGAAAACA TTGCGCATGATGATAAACCGGGCCTGTATTTTCATGAAGAATATGTGGATATGTGCCGCGGCCCGCATGTGCCGAACATGCGCTTTTGCCATCATTTTAAACTGATGAAAACCGCGGGCGCG TATTGGCGCGGCGATAGCAACAACAAAATGCTCCAGCGCATTTATGGCACCGCGTGGGCGGATAAAAAAGCGCTGAACGCGTATCTCCAGCGCCTGGAAGAAGCGGCGAAACGCGATCAT CGCAAAATTGGCAAACAGCTGGATCTGTATCATATGCAGGAAGAAGCGCCGGGCATGGTGTTTTGGCATAACGATGGCTGGACCATTTTTCGCGAACTGGAAGTGTTTGTGCGCAGCAAAC TGAAAGAATATCAGTATCAGGAAGTGAAAGGCCCGTTTATGATGGATCGCGTGCTGTGGGAAAAAACCGGCCATTGGGATAACTATAAAGATGCGATGTTTACCACCAGCAGCGAAAACCG CGAATATTGCATTAAACCGATGAACTGCCCGGGCCATGTGCAGATTTTTAACCAGGGCCTGAAAAGCTATCGCGATCTGCCGCTGCGCATGGCGGAATTTGGCAGCTGCCATCGCAACGAA CCGAGCGGCAGCCTGCATGGCCTGATGCGCGTGCGCGGCTTTACCCAGGATGATGCGCATATTTTTTGCACCGAAGAACAGATTCGCGATGAAGTGAACGGCTGCATTCGCCTGGTGTAT GATATGTATAGCACCTTTGGCTTTGAAAAAATTGTGGTGAAACTGAGCACCCGCCCGGAAAAACGCATTGGCAGCGATGAAATGTGGGATCGCGCGGAAGCGGATCTGGCGGTGGCGCTG GAAGAAAACAACATTCCGTTTGAATATCAGCTGGGCGAAGGCGCGTTTTATGGCCCGAAAATTGAATTTACCCTGTATGATTGCCTGGATCGCGCGTGGCAGTGCGGCACCGTGCAGCTGG ATTTTAGCCTGCCGAGCCGCCTGAGCGCGAGCTATGTGGGCGAAGATAACGAACGCAAAGTGCCGGTGATGATTCATCGCGCGATTCTGGGCAGCATGGAACGCTTTATTGGCATTCTGAC CGAAGAATTTGCGGGCTTTTTTCCGACCTGGCTGGCGCCGGTGCAGGTGGTGATTATGAACATTACCGATAGCCAGAGCGAATATGTGAACGAACTGACCCAGAAACTGAGCAACGCGGG CATTCGCGTGAAAGCGGATCTGCGCAACGAAAAAATTGGCTTTAAAATTCGCGAACATACCCTGCGCCGCGTGCCGTATATGCTGGTGTGCGGCGATAAAGAAGTGGAAAGCGGCAAAGTG GCGGTGCGCACCCGTCGCGGCAAAGATCTGGGCAGCATGGATGTGAACGAAGTGATTGAAAAACTCCAGCAGGAAATTCGCAGCCGCAGCCTGAAACAGCTGGAAGAATAATAATACTAG AGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCT GCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pUC57	Kan	*E. coli* BL21-Gold (DE3)
TrpRS	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGACCAAGCCAATTGTGTTTTCCGGGGCACAACCTTCAGGAGAGTTGACT ATCGGTAATTATATGGGGGCGCTTCGCCAATGGGTTAATATGCAGGATGATTACCACTGCATCTATTGTATTGTAGACCAACATGCTATCACTGTACGCCAAGATGCGCAAAAACTTCGCAAG GCTACCTTAGATACTTTAGCGTTATATTTAGCATGCGGTATCGACCCCGAGAAGTCGACGATTTTCGTCCAGAGCCATGTACCTGAACACGCTCAGTTGGGGTGGGCGCTGAACTGCTATAC TTACTTCGGTGAGCTTTCCCGCATGACCCAGTTCAAGGACAAATCTGCACGCTACGCCGAGAATATTAACGCAGGATTATTTGATTACCCAGTTCTGATGGCAGCCGATATTTTACTGTATCA AACCAACTTGGTACCAGTAGGTGAAGACCAAAAACAACACCTTGAGTTGTCCCGCGACATTGCACAACGTTTCAATGCACTGTATGGAGAAATCTTCAAAGTCCCAGAGCCATTCATCCCTAA AAGCGGGGCTCGCGTGATGTCCTTGTTGGAACCCACTAAGAAAATGAGTAAATCGGACGACAACCGTAACAATGTTATCGGGCTGTTGGAGGATCCTAAATCAGTCGTAAAGAAAATCAAAC GCGCCGTGACGGATAGCGATGAGCCTCCTGTCGTTCGTTACGACGTTCAAAATAAGGCCGGAGTCAGCAATTTGTTAGATATTTTATCAGCTGTCACTGGGCAGTCCATCCCCGAGTTAGAA AAACAATTTGAAGGGAAAATGTACGGTCATCTTAAGGGTGAGGTCGCCGATGCAGTCTCTGGGATGCTTACGGAGCTTCAGGAACGTTACCATCGTTTCCGTAACGACGAGGCCTTCCTGC AACAAGTGATGAAAGATGGCGCCGAGAAAGCGAGCGCCCACGCAAGCCGTACCTTAAAAGCCGTGTACGAGGCTATTGGCTTTGTTGCTAAGCCGGGTGGTAGCGGTGGTGGTAGCGGTC ATCATCATCATCATCATTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACA CTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
TyrRS	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGGCGTCATCGAATTTGATCAAGCAGTTACAGGAACGCGGACTGGTGGC TCAAGTAACGGACGAAGAGGCGTTAGCGGAACGTCTGGCTCAGGGTCCTATTGCGCTGTACTGCGGCTTCGACCCCACAGCGGATTCCCTGCATCTGGGACACTTGGTCCCGTTATTATGC	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-

	TTAAAGCGTTTTCAACAAGCGGGGCACAAGCCGGTCGCTCTGGTTGGAGGCGCAACAGGTTTGATCGGTGACCCTTCCTTCAAGGCTGCTGAACGTAAGCTTAACACGGAAGAGACAGTGC AAGAATGGGTGGATAAAATTCGCAAGCAAGTTGCCCCTTTCTTGGACTTCGACTGTGGGGAAAACTCCGCGATTGCCGCTAACAATTACGATTGGTTCGGAAATATGAACGTGCTTACATTC CTGCGCGATATTGGCAAACACTTTTCTGTAAACCAAATGATTAACAAAGAAGCTGTCAAGCAGCGTTTGAACCGTGAGGACCAGGGTATCTCATTTACAGAATTTTCCTACAATTTACTTCAGG GGTACGATTTTGCATGTTTGAACAAACAATATGGCGTTGTATTACAAATCGGTGGTTCTGACCAATGGGGTAACATTACAAGCGGTATTGACTTAACTCGCCGCCTTCATCAGAACCAAGTGT TCGGGCTGACCGTACCATTAATCACCAAAGCCGATGGTACTAAGTTCGGGAAGACTGAGGGAGGCGCTGTGTGGTTAGACCCAAAAAAGACTTCGCCTTACAAGTTCTATCAGTTTTGGATT AACACGGCGGATGCCGATGTATATCGTTTCTTGAAGTTTTTCACTTTCATGTCCATTGAGGAGATTAATGCTCTGGAGGAGGAGGATAAGAACTCAGGGAAAGCCCCCCCGTGCTCAATATGT ATTAGCAGAACAAGTCACGCGCCTGGTACATGGTGAAGAGGGGCTTCAAGCGGCCAAGCGTATTACCGAGTGCCTGTTCAGTGGCTCCCTGTCGGCCTTATCCGAAGCTGACTTTGAACAG TTAGCACAGGACGGCGTTCCGATGGTTGAAATGGAGAAAGGCGCAGATTTGATGCAAGCTTTGGTAGATTCCGAGCTGCAACCCTCACGCGGCCAGGCTCGCAAAACTATTGCGAGCAATG CCATCACTATCAACGGAGAAAAACAGAGCGATCCAGAATATTTCTTCAAAGAAGAAGACCGTTTATTTGGTCGTTTCACTTTGCTTCGTCGCGGTAAGAAGAATTACTGCTTAATCTGCTGGA AGGGTGGTAGCGGTGGTGGTAGCGGTCATCATCATCATCATCATTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTG TCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3’				
ValRS	5’- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGGAAAAGACTTACAATCCACAAGATATCGAGCAACCCTTGTATGAGCAT TGGGAGAAGCAAGGGTACTTCAAACCCAATGGTGATGAATCACAAGAATCATTTTGCATCATGATTCCTCCCCCGAATGTAACAGGGTCGTTGCATATGGGGCATGCCTTTCAGCAGACGAT CATGGACACAATGATTCGTTATCAACGCATGCAGGGCAAGAACACGTTGTGGCAAGTTGGCACCGATCACGCGGGCATCGCCACGCAGATGGTAGTAGAGCGCAAAATTGCCGCTGAGGA GGGTAAAACACGTCATGATTACGGACGTGAGGCCTTCATTGATAAGATTTGGGAGTGGAAGGCCGAATCGGGAGGTACTATCACTCGTCAAATGCGTCGCTTGGGAAACTCGGTAGACTGG GAACGTGAACGTTTCACGATGGATGAGGGCTTGAGTAATGCGGTGAAAGAGGTGTTTGTCCGCCTGTACAAGGAGGACTTGATCTACCGCGGGAAACGTCTTGTTAATTGGGACCCAAAAC TTCGCACAGCTATTAGTGATCTGGAAGTGGAAAACCGCGAATCTAAAGGCAGTATGTGGCACATTCGCTATCCGTTAGCAGACGGAGCAAAGACCGCCGATGGGAAGGATTACTTGGTGGT TGCCACGACTCGCCCCGAGACTTTACTGGGAGATACTGGTGTTGCTGTGAATCCCGAGGACCCTCGCTACAAAGATTTGATCGGTAAGTACGTTATCCTTCCGTTAGTCAATCGCCGCATCC CGATTGTTGGGGACGAACATGCTGACATGGAAAAGGGGACCGGGTGTGTCAAAATCACCCCCGCCCATGATTTTAACGACTATGAGGTGGGTAAGCGTCATGCTCTGCCCATGATCAACAT TCTTACGTTTGATGGTGACATCCGCGAGAGCGCCCAGGTGTTCGACACAAAAGGGAATGAGTCCGATGTCTATTCTTCAGAGATTCCCGCTGAGTTCCAAAAGCTTGAGCGCTTCGCTGCG CGTAAAGCCGTGGTGGCCGCCGTTGATGCGCTTGGGCTGTTGGAGGAGATCAAGCCACATGATTTGACCGTACCTTACGGGGATCGTGGGGGTGTGGTGATTGAGCCAATGTTGACGGAT CAATGGTATGTCCGCGCTGACGTACTGGCTAAACCAGCCGTCGAGGCCGTAGAGAATGGTGATATCCAGTTTGTCCCAAAGCAATACGAGAACATGTATTTTAGCTGGATGCGCGACATTCA AGACTGGTGTATCTCTCGTCAGTTATGGTGGGGCCACCGTATTCCCGCTTGGTATGATGAGGCTGGCAACGTATACGTGGGCCGTAACGAAGATGAGGTTCGTAAAGAGAACAATCTTGGG GCAGATGTCGTGCTGCGCCAAGATGAAGACGTTTTAGATACTTGGTTCTCGTCGGCTTTGTGGACATTTTCGACGTTAGGTTGGCCAGAAAACACGGATGCCTTACGTCAGTTCCACCCAAC TTCCGTTATGGTAAGCGGTTTCGACATCATTTTTTTCTGGATTGCTCGCATGATCATGATGACGATGCACTTCATCAAAGACGAAAACGGTAAACCTCAGGTACCCTTCCATACGGTGTACAT GACAGGCCTGATTCGTGACGATGAGGGTCAGAAAATGTCAAAATCAAAAGGAAATGTAATTGACCCGCTGGATATGGTCGATGGCATTTCCCTTCCAGAGTTATTGGAAAAGCGCACCGGCA ATATGATGCAGCCGCAACTTGCTGATAAAATCCGTAAACGTACAGAGAAGCAATTTCCGAATGGTATTGAACCGCACGGCACCGACGCGTTGCGCTTCACACTTGCTGCTCTTGCTTCGACT GGACGTGATATTAACTGGGATATGAAGCGTCTTGAAGGCTATCGCAACTTCTGCAATAAATTGTGGAACGCCTCACGTTTTGTCCTGATGAATACAGAAGGTCAGGACTGCGGGTTCAACGG GGGAGAGATGACTCTGAGTTTAGCAGACCGCTGGATTCTTGCGGAATTTAATCAAACGATCAAGGCCTACCGTGAGGCGTTGGATTCTTTTCGTTTTGATATTGCGGCCGGGATCTTATATG AATTTACGTGGAACCAATTTTGCGACTGGTATCTGGAATTAACGAAACCCGTGATGAACGGGGGGACAGAAGCCGAACTTCGTGGGACCCGTCATACTCTTGTCACTGTACTGGAAGGTCT GTTACGTTTGGCACACCCCATTATCCCCTTTATCACAGAAACTATTTGGCAACGCGTAAAGGTTTTATGCGGTATTACGGCAGACACAATTATGTTGCAGCCCTTCCCTCAGTACGACGCCTC TCAAGTGGACGAGGCGGCCTTAGCCGATACAGAGTGGCTTAAGCAAGCGATTGTTGCCGTTCGCAACATCCGTGCGGAGATGAACATCGCTCCGGGGAAGCCACTTGAATTGTTACTGCG CGGTTGCTCAGCCGACGCCGAACGCCGTGTGAATGAGAACCGTGGTTTCTTGCAGACATTGGCGCGCTTAGAATCTATCACTGTGCTTCCTGCGGACGATAAAGGCCCGGTCTCGGTAACT AAAATCATTGACGGCGCAGAGTTGTTAATTCCCATGGCCGGACTTATTAATAAAGAGGATGAGTTAGCACGCTTAGCTAAAGAGGTAGCGAAGATTGAAGGTGAAATTTCCCGCATTGAAAAT AAATTGGCAAATGAAGGATTTGTTGCGCGCGCACCAGAGGCTGTAATTGCGAAGGAACGCGAAAAGTTAGAAGGTTATGCGGAAGCTAAAGCCAAGTTAATTGAGCAACAAGCAGTTATTGC GGCCCTTGGTGGTAGCGGTGGTGGTAGCGGTCATCATCATCATCATCATTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGT TGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3’	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
IF1	5’- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCACCATGGTCACCATCACGGTGGTAGCGGTGGTGGTAGCGGTAT GGCAAAGGAGGATAACATTGAGATGCAGGGTACAGTTTTGGAAACATTACCCAACACCATGTTCCGTGTTGAGCTTGAGAACGGACACGTGGTAACAGCCCACATTTCGGGTAAAATGCGTA AAAATTACATCCGTATCTTAACAGGTGATAAAGTCACTGTAGAACTGACCCCGTATGACCTGAGCAAGGGTCGCATCGTATTCCGCTCTCGCTAATAATACTAGAGCCAGGCATCAAATAAAA CGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGC GGCCGCTGCAG **-3’**	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
IF2	5’- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCACCATGGTCACCATCACGGTGGTAGCGGTGGTGGTAGCGGTAC GGACGTGACGATCAAGACTTTAGCGGCAGAACGTCAGACGTCGGTGGAACGCTTAGTCCAACAATTCGCAGACGCTGGTATTCGCAAATCTGCGGACGATTCAGTGTCGGCCCAAGAGAA GCAAACGTTGATTGATCACTTGAATCAGAAGAACTCGGGACCGGACAAGTTGACATTGCAACGCAAAACACGTTCCACACTGAACATTCCCGGCACCGGTGGCAAAAGCAAATCGGTCCAG ATCGAAGTCCGCAAGAAACGCACATTCGTGAAGCGTGATCCTCAGGAAGCCGAGCGTCTGGCCGCAGAGGAGCAAGCGCAACGTGAAGCAGAGGAACAGGCGCGTCGTGAGGCCGAAGA GTCTGCGAAACGCGAAGCCCAACAGAAGGCAGAGCGCGAGGCCGCTGAACAAGCGAAGCGTGAAGCAGCTGAGCAGGCAAAACGCGAAGCTGCTGAGAAAGATAAAGTTAGCAATCAGC AGGATGACATGACGAAGAACGCCCAAGCCGAGAAGGCACGTCGTGAACAGGAAGCAGCAGAGCTGAAGCGTAAGGCTGAGGAGGAGGCGCGTCGCAAATTGGAAGAAGAGGCACGTCG TGTGGCGGAGGAGGCTCGTCGCATGGCAGAGGAAAACAAATGGACAGATAATGCTGAACCTACCGAAGACTCTAGTGATTACCACGTTACTACGTCTCAACACGCCCGCCAAGCAGAGGAT GAGAGTGATCGTGAGGTCGAGGGGGGACGCGGTCGCGGACGCAATGCTAAAGCAGCGCGCCCCAAAAAGGGCAATAAACATGCTGAGTCAAAGGCGGACCGTGAGGAGGCTCGTGCTG CGGTCCGCGGTGGTAAAGGCGGCAAGCGTAAAGGCTCTTCATTACAGCAGGGATTTCAGAAACCTGCTCAGGCGGTTAACCGCGACGTGGTAATTGGTGAGACCATCACAGTGGGAGAGT TAGCCAATAAAATGGCCGTGAAGGGTTCTCAAGTGATCAAGGCAATGATGAAGCTGGGGGCGATGGCAACGATCAACCAAGTAATTGACCAGGAAACCGCCCAACTGGTCGCTGAGGAGA TGGGACACAAGGTTATCTTACGTCGCGAAAACGAGCTGGAGGAGGCGGTAATGTCGGATCGCGATACGGGGGCCGCAGCGGAACCCCGCGCGCCCGTGGTGACTATTATGGGACATGTA GACCATGGAAAGACGTCACTGTTAGACTACATTCGTAGTACTAAGGTTGCCTCTGGTGAGGCTGGCGGAATCACGCAGCACATCGGCGCCTACCATGTGGAAACCGAGAACGGTATGATCA CTTTCTTGGATACCCCAGGTCATGCAGCCTTTACATCGATGCGCGCTCGTGGGGCACAGGCAACCGATATTGTAGTGTTAGTGGTCGCAGCTGACGATGGTGTGAGTCCTCAAACTATCGA GGCCATTCAACATGCGAAGGCTGCGCAGGTGCCTGTAGTTGTGGCCGTTAATAAAATTGATAAACCCGAGGCCGATCCAGACCGCGTAAAGAACGAACTTTCACAGTATGGAATCCTTCCT GAGGAGTGGGGAGGAGAGTCTCAGTTCGTACACGTTAGTGCAAAGGCCGGCACGGGGATTGACGAGTTATTAGACGCGATTCTTTTGCAGGCAGAGGTTTTGGAGCTGAAAGCCGTACGT AAGGGAATGGCGTCCGGGGCTGTGATCGAAAGCTTTCTTGATAAAGGACGTGGCCCAGTCGCAACAGTCTTGGTGCGCGAGGGGACCTTACATAAGGGTGATATCGTTTTGTGCGGTTTC	*E. coli*	pJET	Amp	*E. coli* BL21-Gold (DE3)

	GAATATGGCCGTGTGCGCGCTATGCGCAATGAGCTGGGGCAGGAGGTACTTGAGGCTGGGCCGTCCATTCCAGTAGAAATCTTGGGGCTTTCCGGTGTCCCCGCCGCCGGGGACGAAGT AACCGTAGTACGCGACGAGAAGAAAGCCCGTGAGGTGGCCTTATACCGCCAGGGTAAGTTCCGCGAAGTGAAACTTGCCCGCCAACAAAAGAGCAAACTGGAAAATATGTTTGCTAATATG ACGGAGGGCGAAGTTCACGAGGTTAACATTGTTTTAAAAGCCGATGTACAAGGAAGCGTTGAAGCTATTTCCGACTCACTGTTAAAGTTGAGCACCGACGAGGTTAAAGTGAAGATCATTGG TAGCGGGGTAGGCGGAATCACTGAGACAGATGCAACATTAGCAGCCGCTTCGAATGCGATCTTGGTCGGTTTCAACGTCCGTGCTGATGCTAGCGCTCGTAAAGTTATCGAAGCTGAGAGT TTAGACCTTCGTTACTATTCGGTAATTTACAATTTAATCGACGAGGTTAAGGCTGCAATGTCGGGAATGCTTAGCCCCGAGTTAAAACAACAAATTATCGGTTTAGCTGAGGTACGCGATGTTT TCAAGAGTCCGAAGTTTGGTGCCATCGCCGGTTGCATGGTTACAGAAGGGGTTGTCAAACGTCACAATCCCATTCGTGTTTTACGTGACAATGTTGTTATTTACGAGGGGGAGTTAGAGTCA CTTCGCCGCTTTAAAGATGAGATGTTAATGAGGTTCGTAATGGAATGGAATGTGGGATCGGAGTGAAGAACTATAATGACGTGCGTACCGGCGATGTAATCGAGGTGTTTGAAATCATTGAAATT CAACGTACAATCGCATAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACAC TGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'				
IF3	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCACCATGGTCATCCATCACGGTGGTAGCGGTGGTGGTAGCGGTA AGGGTGGAAAGCGCGTACAGACCGCACGCCCAAACCGTATCAACGGTGAAATTCGTGCACAAGAGGTTCGTTTGACGGGGTTAGAGGGTGAACAGTTGGGGATCGTGTCATTACGCGAGG CGTTGGAAAAGGCTGAAGAAGCGGGGGTCGACTTAGTCGAAATTAGCCCCAATGCGGAGCCGCCGGTTTGCCGTATTATGGATTATGGCAAGTTCTTATATGAGAAAAGTAAGAGCAGTAA AGAGCAAAAGAAGAAGCAAAAAGTTATCCAGGTCAAAGAAATTAAATTTCGTCCAGGTACGGATGAGGGAGACTACCAGGTCAAGTTACGTTCCCTTATTCGTTTCTTAGAAGAGGGCGACA AGGCAAAGATCACGTTGCGCTTCCGCGGGCGTGAAATGGCCCACCAACAAATTGGGATGGAAGTTCTGAATCGCGTAAAAGATGACTTGCAAGAATTGGCCGTCGTCGAATCCTTCCCGAC AAAGATTGAGGGGCGTCAGATGATTATGGTCCTGGCACCTAAGAAAAGCAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGT TGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
EF-G	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCATCATCATCATCATGGTGGTAGCGGTGGTGGTAGCGGTGCACG TACCACCCCGATTGCACGTTATCGTAATATTGGTATTAGCGCACATATTGATGCAGGTAAAACCACCACCACCGAACGTATTCTGTTTTATACCGGTGTTAATCATAAAATTGGTGAAGTTCAT GATGGTGCAGCAACCATGGATTGGATGGAACAGGAACAGGAACGTGGTATTACCATTACCAGCGCAGCAACCACCGCATTTTGGAGCGGTATGGCAAAACAGTATGAACCGCATCGTATTA ATATTATTGATACCCCGGGTCATGTTGATTTTACCATTGAAGTTGAACGTAGCATGCGTGTTCTGGATGGTGCAGTTATGGTTTATTGTGCAGTTGGTGGTGTTCAGCCGCAGAGCGAAACCG TTTGGCGTCAGGCAAATAAATATAAAGTTCCGCGTATTGCATTTGTTAATAAAATGGATCGTATGGGTGCAAATTTTCTGAAAGTTGTTAATCAGATTAAAACCCGTCTGGGTGCAAATCCGGT TCCGCTTCAACTGGCAATTGGTGCAGAAGAACATTTTACCGGTGTTGTTGATCTGGTTAAAATGAAAGCAATTAATTGGAATGATGCAGATCAGGGTGTTACCTTTGAATATGAAGATATTCCG GCAGATATGGTTGAACTGGCAAATGAATGGCATCAGAATCTGATTGAAAGCGCAGCAGAAGCAAGCGAAGAACTGATGGAAAAATATCTGGGTGGTGAAGAACTGACCGAAGCAGAAATTA AAGGTGCACTGCGTCAGCGTGTTCTGAATAATGAAATTATTCTGGTTACCTGTGGTAGCGCATTTAAAAATAAAGGTGTTCAGGCAATGCTGGATGCAGTTATTGATTATCTGCCGAGCCCGG TTGATGTTCCGGCAATTAATGGTATTCTGGATGATGGTAAAGATACCCCGGCAGAACGTCATGCAAGCGATGATGAACCGTTTAGCGCACTGGCATTTAAAATTGCAACCGATCCGTTTGTTG GTAATCTGACCTTTTTTCGTGTTTATAGCGGTGTTGTTAATAGCGGTGATACCGTTCTGAATAGCGTTAAAGCAGCACGTGAACGTTTTGGTCGTATTGTTCAGATGCATGCAAATAAACGTGA AGAAATTAAAGAAGTTCGTGCAGGTGATATTGCAGCAGCAATTGGTCTGAAAGATGTTACCACCGGTGATACCCTGTGTGATCCGGATGCACCGATTATTCTGGAACGTATGGAATTTCCGG AACCGGTTATTAGCATTGCAGTTGAACCGAAAACCAAAGCAGATCAGGAAAAAATGGGTCTGGCACTGGGTCGTCTGGCAAAAGAAGATCCGAGCTTTCGTGTTTGGACCGATGAAGAAAG CAATCAGACCATTATTGCAGGTATGGGTGAACTGCATCTGGATATTATTGTTGATCGTATGAAACGTGAATTTAATGTTGAAGCAAATGTTGGTAAACCGCAGGTTGCATATCGTGAACCATT CGTCAGAAAGTTACCGATGTTGAAGGTAAACATGCAAAACAGAGCGGTGGTCGTGGTCAGTATGGTCATGTTGTTATTGATATGTATCCGCTGGAACCGGGTAGCAATCCGAAAGGTTATGA ATTTATTAATGATATTAAAGGTGGTGTTATTCCGGGTGAATATATTCCGGCAGTTGATAAAGGTATTCAGGAACAGCTGAAAGCAGGTCCGCTGGCAGGTTATCCGGTTGTTGATATGGGTAT TCGTCTGCATTTTGGTAGCTATCATGATGTTGATAGCAGCGAACTGGCATTTAAACTGGCAGCAAGCATTGCATTTAAAGAAGGTTTTAAAAAAGCAAAACCGGTTCTGCTGGAACCGATTAT GAAAGTTGAAGTTGAAACCCCGGAAGAAAATACCGGTGATGTTATTGGTGATCTGAGCCGTCGTCGTGGTATGCTGAAAGGTCAGGAAAGCGAAGTTACCGGTGTTAAAATTCATGCAGAAG TTCCGCTGAGCGAAATGTTTGGTTATGCAACCCAGCTGCGTAGCCTGACCAAAGGTCGTGCAAGCTATACCATGGAATTTCTGAAATATGATGAAGCACCGAGCAATGTTGCACAGGCAGTT ATTGAAGCACGTGGTAAATAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCA CACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG-3'	*E. coli*	pET28a	Amp	*E. coli* BL21-Gold
EF-Tu	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCACCATGGTCACCATCACGGTGGTAGCGGTGGTGGTAGCGGTTC GAAGGAAAAGTTCGAACGCACTAAGCCACACGTCAATGTTGGGACCATCGGTCACGTTGATCACGGGAAGACTACTCTTACGGCCGCAATCACAACCGTATTGGCTAAGACCTACGGTGGG GCCGCCCGCGCTTTCGACCAAATTGACAACGCACCGGAAGAGAAGGCGCGCGGCATTACGATCAACACCTCCCATGTTGAGTACGATACGCCAACTCGTCATTACGCGCACGTAGATTGTC CCGGCCACGCGGATTACGTAAAAAATATGATCACCGGAGCTGCTCAGATGGATGGGGCGATTCTGGTGGTCGCTGCTACTGACGGCCCAATGCCGCAAACGCGCGAGCACATCTTGTTAG GTCGCCAGGTTGGAGTCCCCTACATCATTGTATTTCTGAATAAATGTGACATGGTTGATGATGAAGAACTGCTGGAACTGGTTGAGATGGAAGTTCGTGAATTGCTGTCCCAATACGATTTCC CCGGTGACGACACCCCGATTGTACGTGGGTCTGCCTTAAAAGCCCTTGAGGGTGATGCAGAGTGGGAAGCGAAGATCCTTGAATTAGCTGGCTTTCTGGATTCGTACATCCCAGAGCCTGA ACGCGCTATTGACAAGCCTTTTTTACTGCCGATTGAGGACGTTTTCTCTATTTCCGGTCGCGGGACTGTCGTAACTGGTCGTGTGGAACGCGGCATCATCAAGGTTGGCGAAGAAGTAGAAA TCGTAGGCATCAAGGAGACTCAGAAAAGCACCTGTACGGGCGTGGAGATGTTTCGCAAGTTGCTGGACGAGGGGCGTGCAGGCGAGAATGTTGGCGTGCTGTTGCGTGGTATCAAGCGT GAGGAGATCGAACGTGGACAGGTATTAGCAAAGCCGGGCACCATCAAACCCCATACAAAATTCGAAAGCGAAGTCTATATTTTATCAAAAGATGAGGGTGGTCGTCACACGCCATTTTTTAA AGGATACCGCCCGCAGTTTTACTTTCGCACCACGGACGTTACAGGTACTATTGAGCTTCCGGAGGGTGTGGAAATGGTCATGCCTGGCGACAACATTAAGATGGTTGTCACTTTAATTCATC CTATTGCGATGGATGACGGCTTACGTTTCGCCATCCGCGAAGGTGGCCGTACCGTAGGAGCGGGAGTGGTTGCTAAAGTTTTAGGATAATAATACTAGAGCCAGGCATCAAATAAAACGAA AGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCC GCTGCAG-3'	*E. coli*	pET21a	Amp	*E. coli* BL21-Gold
EF-Ts	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGGCGGAAATCACAGCTAGCCTTGTCAAAGAATTACGCGAGCGTACTGG GGCCGGAATGATGGATTGTAAAAAGGCGCTTACAGAGGCCAACGGTGACATTGAGCTTGCCATTGAGAACATGCGTAAATCTGGAGCGATTAAAGCAGCAAAAAGGCCGGTAACGTAGCC GCTGACGGAGTGATTAAAACGAAAATCGATGGTAACTATGGCATCATCCTGGAGGTAAATTGTCAAACAGATTTTGTTGCGAAGGACGCTGGATTTCAAGCCTTCGCCGATAAAGTCCTTGA CGCAGCAGTCGCTGGTAAGATTACTGATGTTGAGGTGCTGAAAGCGCAATTCGAGGAGGAGCGTGTTGCTCTTGTGGCAAAGATCGGAGAAAATATTAACATTCGCCGCGTGGCCGCGCTT GAGGGCGATGTCCTTGGCTCATATCAGCATGGAGCACGTATCGGCGTTTTGGTAGCAGCTAAGGGGGCGGACGAGGAACTGGTAAAACATATCGCCATGCACGTAGCCGCTTCGAAGCCG GAATTTATTAAACCCGAAGATGTGTCCGCTGAAGTTGTCGAAAAGGAATACCAAGTGCAACTTGACATTGCCATGCAGAGCGGCAAACCAAAAGAAATCGCAGAGAAAATGGTGGAGGGAC GTATGAAAAAATTCACGGGGGAAGTATCCTTGACGGGGCAGCCGTTCGTTATGGAGCCATCCAAAACCGTGGGTCAGTTACTGAAAGAACATAATGCAGAGGTCACAGGTTTCATCCGCTTT GAGGTGGGCGAAGGCATTGAGAAAGTTGAGACAGATTTTGCAGCAGAGGTAGCTGCAATGTCTAAACAGAGTGGTGGTAGCGGTGGTGGTAGCGGTCATCATCATCATCATTAATAAT	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)

	ACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCC TTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3’				
RF1	5’- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCATCATCATCATCATGGTAGCGGTGGTGGTAGCGGTGGTAAACC GAGCATTGTGGCGAAACTGGAAGCGCTGCATGAACGCCATGAAGAAGTGCAGGCGCTGCTGGGCGATGCGCAGACCATTGCGGATCAGGAACGCTTTCGCGCGCTGAGCCGCGAATATG CGCAGCTGAGCGATGTGAGCCGCTGCTTTACCGATTGGCAGCAGGTGCAGGAAGATATTGAAACCGCGCAGATGATGCTGGATGATCCGGAAATGCGCGAAATGGCGCAGGATGAACTGC GCGAAGCGAAAGAAAAAAGCGAACAGCTGGAACAGCAGTTACAGGTGCTGCTGCTGCCGAAAGATCCGGATGATGAACGCAACGCGTTTCTGGAAGTGCGCGCGGGCACCGGCGGCGAT GAAGCGGCGCTGTTTGCGGGCGATCTGTTTCGCATGTATAGCCGCTATGCGGAAGCGCGCCGCTGGCGCGTGGAAATTATGAGCGCGAGCGAAGGCGAACATGGCGGCTATAAAGAAAT TATTGCGAAAATTAGCGGCGATGGCGTGTATGGCCGCCTGAAATTTGAAAGCGGCGGCCATCGCGTGCAGCGCGTGCCGGCGACCGAAAGCCAGGGCCGCATTCATACCAGCGCGTGCA CCGTGGCGGTGATGCCGGAACTGCCGGATGCGGAACTGCCGGATATTAACCCGGCGGATCTGCGCATTGATACCTTTCGCAGCAGCGGCGCGGGCGGCCAGCATGTGAACACCACCGAT AGCGCGATTCGCATTACCCATCTGCCGACCGGCATTGTGGTGGAATGCCAGGATGAACGCAGCCAGCATAAAAACAAAGCGAAAGCGCTGAGCGTGCTGGGCGCGCGCATTCATGCGGC GGAAATGGCGAAACGCCAGCAGGCGGAAGCGAGCACCCGCCGCAACCTGCTGGGCAGCGGCGATCGCAGCGATCGCAACCGCACCTATAACTTTCCGCAGGGCCGCGTGACCGATCAT CGCATTAACCTGACCCTGTATCGCCTGGATGAAGTGATGGAAGGCAAACTGGATATGCTGATTGAACCGATTATTCAGGAACATCAGGCGGATCAGCTGGCGGCGCTGAGCGAACAGGAAT AATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGT GGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3’	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
RF2	5’- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCACCATGGTCACCATCACGGTGGTAGCGGTGGTGGTAGCGGTTT TGAAATCAATCCTGTAAACAACCGCATCCAAGACTTGACGGAACGCTCAGATGTGCTTCGCGGTTACCTGGACTATGATGCAAAGAAGGAGCGTTTAGAAGAAGTAAATGCCGAGCTTGAGC AGCCAGATGTATGGAACGAGCCGGAGCGCGCTCAGGCGTTGGGGAAAGAGCGTAGCTCCTTAGAGGCCGTGGTTGATACCTTAGATCAAATGAAACAGGGCCTTGAGGATGTGAGTGGCC TGTTAGAGCTTGCTGTTGAAGCAGATGATGAGGAAACTTTCAACGAGGCCGTAGCCGAACTTGATGCTCTTGAGGAAAAACTGGCTCAGCTGGAGTTTCGTCGTATGTTTTCAGGTGAGTAT GACTCAGCTGATTGTTACTTAGACATCCAGGCCGGAAGTGGCGGAACGGAGGCGCAGGATTGGGCTAGTATGCTGGAACGTATGTATCTTCGTTGGGCAGAGTCACGCGGCTTCAAAACT GAGATTATCGAAGAGTCCGAGGGAGAAGTCGCCGGTATCAAGTCCGTCACAATTAAGATTTCCGGTGATTATGCCTACGGCTGGTTGCGTACGGAGACTGGGGTCCACCGTTTGGTCCGCA AGAGCCCGTTCGATTCAGGAGGGCGTCGTCATACTAGCTTCTCTTCAGCTTTCGTCTACCCAGAGGTGGATGATGACATCGATATTGAAATCAACCCCGCCGACCTTCGCATCGACGTGTAT CGCACATCCGGTGCAGGAGGACAGCATGTAAACCGTACAGAGTCTGCGGTCCGCATCACTCATATCCCAACAGGCATCGTCACCCAGTGCCAGAACGACCGTTCGCAGCATAAGAATAAG GATCAGGCTATGAAGCAGATGAAGGCTAAGTTATATGAGTTAGAGATGCAGAAAAAAAAATGCGGAAAAACAGGCAATGGAAGATAACAAGAGCGACATCGGGTGGGGTTCCCAGATTCGTT CCTACGTTTTAGACGACAGCCGCATCAAGGACCTTCGCACAGGGGTCGAGACACGTAATACCCAAGCCGTTTTGGACGGGAGCCTTGACCAATTTATTGAGGCTTCGCTGAAGGCGGGACT TTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGG GTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3’	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
RF3	5’- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCATCATCATCATCATGGTGGTAGCGGTGGTGGTAGCGGTACTCTG AGCCCTTATCTGCAAGAAGTCGCGAAACGTCGCACATTTGCGATCATCTCGCATCCGGATGCCGGTAAGACGACGATTACGGAAAAGGTGCTGCTTTTTGGTCAAGCTATTCAAACGGCAG GTACCGTCAAGGGACGCGGAAGCAACCAACATGCAAAATCAGACTGGATGGAGATGGAAAAACAGCGCGGGATCTCGATCACCACGTCCGTAATGCAGTTTCCATATCACGACTGTCTGGT CAACTTACTGGATACTCCAGGACACGAAGATTTTTCTGAGGATACCTATCGTACCTTAACTGCCGTAGATTGCTGTTTAATGGTAATCGACGCTGCGAAGGGGGTAGAAGACCGTACACGTA AATTGATGGAGGTCACACGCCTTCGTGATACGCCCATTTTAACCTTTATGAACAAACTGGACCGTGACATCCGCGATCCCATGGAGCTGCTGGACGAAGTAGAAAATGAGTTAAAAATCGGC TGTGCGCCAATTACCTGGCCTATCGGTTGTGGGAAATTGTTCAAAGGAGTGTATCATCTTTATAAAGACGAGACGTACTTGTACCAAAGTGGAAAGGGACATACGATCCGAGGAAGTTCGCAT CGTGAAAGGCTTAAATAATCCGGATCTTGACGCTGCGGTAGGAGAAGACTTGGCTCAGCAGTTACGCGACGAACTGGAGTTAGTTAAAGGTGCTTCGAATGAATTTGATAAAGAGCTTTTCT TAGCAGGAGAGATCACTCCAGTCTTCTTTGGGACTGCTTTAGGCAACTTTGGGGTGGACCATATGCTTGACGGGTTGGTTGAGTGGGCGCCGGCTCCGATGCCCCGTCAAACAGATACTCG TACAGTTGAAGCATCGGAAGACAAGTTTACCGGTTTTGTATTCAAAATCCAAGCGAACATGGACCCTAAGCATCGTGATCGCGTTGCCTTCATGCGTGTTGTATCAGGTAAGTATGAGAAAG GAATGAAACTTCGTCAAGTGCGTACGGCTAAGGACGTGGTTATTAGTGATGCGTTGACATTCATGGCAGGAGACCGTTCCCATGTTGAGGAAGCTTACCCAGGAGATATTCTGGGATTACAT AATCACGGAACAATTCAGATTGGGGACACATTCACGCAAGGTGAGATGATGAAATTCACTGGGATTCCTAATTTCGCGCCTGAGCTTTTTCGCCCGCATTCGCCTGAAAGACCCATTAAAGCA AAAACAACTTTTGAAAGGGTTAGTACAATTGTCCGAGGAGGGTGCAGTTCAAGTGTTTCGTCCGATTTCAAATAACGATTTAATTGTTGGTGCGGTCGGCGTCCTTCAGTTCGATGTTGTTGT GGCTCGTCTTAAATCAGAGTACAACGTCGAAGCCGTGTACGAGAGCGTGAACGTTGCCACGGCGCGTTGGGTCGAATGTGCGGACGCGAAGAAGTTCGAAGAGTTTAAACGTAAAAATGA GAGTCAATTGGCGTTGGACGGAGGGGACAAACCTGGCTTATATCGCGACTTCGATGGTGAATTTACGCTTAGCTCAAGAGCGTTACCCAGATGTCCAGTTCCACCAAACCCGTGAGCACTAA TAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTG GGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3’	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
MTF	5’- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGTCCGAGAGTTTGCGTATCATTTTCGCGGGGACCCCTGATTTTGCGGCC CGCCACCTGGATGCGTTACTTTCCTCCGGCCATAACGTTGTTGGGGTATTCACACAACCTGATCGTCCGGCGGGGCGTGGTAAGAAGCTGATGCCTTCTCCGGTCAAAGTCCTGGCGGAG GAAAAGGGATTGCCTGTCTTTCAACCGGTGAGTTTACGTCCACAAGAGAATCAACAGTTGGTAGCGGAACTTCAGGCTGACGTGATGGTTGTTGTAGCTTACGGTCTTATCTTACCAAAAGC GGTCTTGGAGATGCCACGTCTGGGTTGCATCAATGTACACGGGTCCTTACTTCCTCGTTGGCGCGGCGCAGCACCCATTCAACGCAGCTTGTGGGCGGGCGACGCCGAGCTTGGCGTCA CCATTATGCAAATGGACGTAGGTCTGGACACGGGCGATATGCTGTACAAACTGTCTTGTCCGATCACCGCGGAGGATACGTCCGGGACGTTATACGACAAGTTAGCAGAATTGGGACCCCA AGGCCTTATCACTACGTTAAAACAACTTGCTGATGGGACGGCAAAACCTGAGGTACAAGATGAGACGTTGGTGACTTATGCCGAGAAATTAAGTAAAGAGGAGGCGCGCATTGACTGGAGC TTATCCGCGGCACAACTTGAACGCTGCATTCGTGCATTTAATCCGTGGCCGATGAGCTGGCTTGAAATCGAGGGTCAACCCGTCAAGGTTTGGAAAGCTAGTGTGATTGATACCGCCACTAA TGCGGCCCCGGGAACAATTTTAGAGGCCAATAAACAGGGGATTCAAGTTGCCACGGGGGATGGGATCCTTAACTTGCTTTCATTACAGCCTGCGGGCAAAAAAGCTATGTCAGCGCAGGAC TTGCTTAACTCACGTCGTGAGTGGTTTGTCCCCGGGAACCGTCTGGTTGGTGGTAGCGGTGGTGGTAGCGGTCATCATCATCATCATTAAATAACTAGAGCCAGGCATCAAATAAAAC GAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCG GCCGCTGCAG -3’	*E. coli*	pJET	Amp	*E. coli* BL21-Gold (DE3)
RRF	5’- GAATTCGGCCGCTTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGATTTCAGATATTCGCAAAGATGCTGAAGTTCGTATGGACAAGTGTGTGGAAG CGTTTAAGACTCAAATTTCCAAAATTCGCACCGGACGCGCGTCGCCTTCGTTATTAGACGGCATCGTAGTAGAGTATTACGGTACGCCCACACCCCTGCGTCAGTTGGCCTCGGTTACCGTC GAAGATAGCCGCACGCTTAAAATCAATGTTTTTGATCGCTCGATGTCACCCGCAGTAGAAAAGGCAATTATGGCGTCAGACCTGGGACTGAACCCGAACTCGGCTGGGTCTGATATCCGCG	*E. coli*	pJET	Amp	*E. coli* BL21-Gold (DE3)

	TTCCACTTCCTCCATTAACTGAGGAACGCCGCAAGGACCTTACAAAGATCGTACGCGGCGAGGCCGAACAGGCCCGCGTTGCCGTTCGCAATGTCCGCCGTGACGCGAATGACAAAGTCA AGGCACTTTTAAAGGATAAAGAAATTTCGGAGGACGACGACCGCCGCAGTCAGGATGACGTGCAGAAACTTACTGACGCAGCTATCAAGAAGATTGAGGCTGCCCTTGCCGACAAGGAGG CTGAACTTATGCAGTTCGGTGGTAGCGGTGGTGGTAGCGGTCATCACCATGGTCACCATCACTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCT TTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'				
MK	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCACCATGGTCACCATCACGGTGGTAGCGGTGGTGGTAGCGGTCG TATCATTCTTTTGGGTGCTCCTGGTGCAGGGAAAGGAACCCAGGCGCAGTTCATCATGGAAAAGTACGGTATCCCCCAGATTTCTACGGGAGATATGTTGCGTGCCGCTGTAAAGTCGGGC AGTGAATTGGGTAAACAGGCTAAGGATATCATGGATGCTGGTAAACTGGTGACGGACGAATTAGTCATTGCGTTGGTTAAAGAGCGTATTGCCCAGGAGGATTGTCGCAATGGGTTCTTATT AGACGGTTTCCCCCGCACAATTCCGCAGGCTGACGCTATGAAGGAGGCCGGAATCAATGTTGATTATGTTTTAGAATTTGACGTACCTGATGAATTGATTGTCGACCGCATTGTGGGTCGTC GCGTTCACGCGCCCTCGGGTCGCGTCTACCATGTGAAGTTTAACCCTCCGAAAGTCGAGGGCAAAGACGATGTCACCGGAGAGGAGTTGACAACTCGCAAAGATGACCAGGAAGAGACAG TTCGCAAACGTCTGGTTGAGTATCATCAGATGACGGCCCCCTTAATCGGATACTACTCTAAGGAGGCCGAGGCTGGAAATACTAAGTATGCGAAGGTCGACGGTACAAAGCCTGTAGCCGA AGTCCGCGCAGATTTGGAAAAGATCTTGGGCTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCT CTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pSB1C3	Chlor	E. coli BL21-Gold (DE3)
CK	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCCCTTCGGAAACACGCATAACAAGTACAAATTGAACTATAAGAGTGAG GAGGAATATCCCGACCTTAGTAAACACAACAACCACATGGCGAAGGTCTTGACACCCGACTTATATAAAAAATTGCGCGATAAAGAGACACCTTCCGGGTTTACTTTAGACGACGTCATCCA GACGGGCGTGGATAATCCTGGACACCCCTTCATTATGACTGTTGGATGTGTTGCCGGAGACGAAGAAAGCTACACCGTATTTAAGGATCTTTTCGATCCCATTATCCAGGATCGGTCATGGAG GGTTCAAGCCGACCGACAAACACAAGACGGATTTAAACCATGAAAACCTTAAAGGCGGAGATGACTTAGATCCCCACTACGTTTTGTCAAGTCGTGTGCGTACTGGTCGCTCCATCAAAGGG TACACTCTTCCTCCTCATTGTAGCCGTGGCGAGCGCCGCGCCGTAGAAAAGTTATCGGTGGAGGCGTTAAATTCTCTGACGGGAGAGTTCAAGGGAAAATACTACCCGTTGAAGTCAATGA CGGAACAAGAACAACAGCAATTGATCGATGACCACTTTCTGTTCGATAAACCCGTCTCGCCCCTTCTGCTGGCGTCGGGTATGGCACGTGACTGGCCGGATGCACGTGGTATTTGGCACAA CGACAATAAGTCCTTTTTAGTATGGGTTAATGAGGAAGATCATTTACGCGTTATTTCAATGGAAAAGGGCGGTAACATGAAAGAAGTTTTCCGCCGTTTCTGTGTGGGGTTACAGAAGATCGA GGAGATTTTCAAGAAAGCTGGTCATCCATTCATGTGGAATGAACACCTTGGTTACGTATTAACGTGCCCTTCTAATCTGGGTACCGGTTTGCGCGGTGGTGTTCATGTCAAATTGGCCCACTT GTCGAAGCATCCGAAATTTGAGGAAATTCTTACTCGTTTGCGCTTACAAAAACGTGGGACTGGCGGAGTAGACACAGCCGCAGTAGGTTCTGTATTTGATATCTCGAATGCCGACCGTCTTG GGTCCTCAGAGGTCGAACAAGTGCAGTTGGTGGTCGATGGGGTCAAGCTTATGGTTGAGATGGAGAAGAAACTTGAAAAAGGCCAAAGTATCGATGATATGATCCCGGCCCAGAAAGGTG GTAGCGGTGGTGGTAGCGGTCATCATCATCATCATTAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTG AACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	Rabbit	N/A	N/A	N/A
NDK	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCACCATGGTCACCATCACGGTGGTAGCGGTGGTGGTAGCGGTCA CCACTCGTCCGGTCTGGTCCCCCGTGGTTCCCATATGGGCTCTCACATGGCGATTGAGCGTACATTTAGTATCATTAAGCCGAATGCGGTGGCGAAAAATGTTATCGGCAATATTTTTGCTC GCTTTGAAGCAGCCGGATTCAAAATTGTAGGGACGAAGATGCTGCATTTGACCGTGGAACAGGCGCGTGGTTTCTATGCCGAGCATGACGGAAAACCCTTTTTTGACGGGCTTGTTGAGTTT ATGACGTCGGGCCCCATTGTGGTGTCGGTTTTGGAGGGAGAAAACGCAGTACAACGCCATCGTGATTTGCTGGGGGCCACTAACCCAGCCAATGCATTGGCCGGAACTTTACGCGCGGAC TATGCAGACTCGCTGACGGAAAATGGGACACACGGCTCCGATAGCGTCGAGAGCGCCGCCCGTGAGATTGCATACTTCTTTGGTGAGGGGGAAGTCTGTCCCCGTACGCGCTAATAATAC TAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTT TCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'	*E. coli*	pSB1C3	Chlor	*E. coli* BL21-Gold (DE3)
PPiase	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCACCATGGTCACCATCACGGTGGTAGCGGTGGTGGTAGCGGTAA GGTTGCTAAAGACTTAGTGGTCAGCCTGGCCTACCAAGTCCGCACGGAGGATGGAGTTTTAGTTGACGAGTCTCCAGTATCTGCCCCTTTGGATTACTTGCATGGACATGGTAGCTTAATTA GCGGTCTGGAAACCGCCTTGGAGGGGCACGAAGTGGGTGATAAATTCGATGTTGCTGTTGGTGCCAATGACGCTTATGGCCAGTACGATGAAAACTTAGTGCAGCGCGTCCCAAAGGATG TTTTCATGGGGGTTGATGAATTACAGGTAGGTATGCGCTTCCTTGCAGAGACGGATCAAGGCCCTGTGCCAGTTGAGATCACGGCGGTGGAAGATGACCACGTAGTCGTAGACGGCAATCA TATGCTTGCAGGGCAGAACTTAAAATTCAACGTGGAAGTAGTTGCGATCCGCGAAGCTACGGAGGAAGAACTTGCTCATGGACACGTTCACGGGGCCCACGACCATCACCACGACCACGAT CACGATGGTTGTTGTGGCGGGCATGGGCACGACCATGGTCATGAACACGGGGGGGAAGGGTGTTGCGGGGGAAAGGGGAATGGTGGCTGCGGCTGCCATTAATAATACTAGAGCCAGGC ATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATA TACTAGTAGCGGCCGCTGCAG -3'	*Saccharomyces cerevisiae*	pSB1C3	Chlor	E. coli BL21-Gold (DE3)
T7 RNAP	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGCATCACCATGGTCACCATCACGGTGGTAGCGGTGGTGGTAGCGGTAA CACGATCAATATTGCGAAGAATGACTTTAGCGACATCGAGCTTGCAGCTATTCCTTTCAACACTTTAGCGGACCACTATGGGGAGCGTTTGGCTCGCGAGCAGTTAGCCCTTGAACATGAGA GCTATGAGATGGGCGAAGCTCGCTTCCGCAAAATGTTCGAGCGTCAGTTGAAAGCCGGCGAAGTCGCTGATAACGCAGCTGCGAAGCCTCTGATCACCACGTTACTGCCAAAAATGATCGC GCGTATCAATGATTGGTTCGAAGAGGTGAAGGCAAAGCGCGGTAAACGTCCGACAGCATTTCAATTCTTGCAAGAGATTAAACCGGAGGCCGTTGCATACATCACGATCAAAACGACTCTTG CTTGTTTAACTTCAGCTGATAATACCACTGTCCAGGCGGTAGCATCCGCCATCGGTCGTGCCATCGAAGACGAAGCGCGCTTTGGCCGTATTCGTGATTTGGAAGCTAAACATTTTAAGAAA AACGTGGAGGAGCAATTAAATAAACGCGTTGGACATGTGTATAAGAAGGCCTTTATGCAGGTAGTAGAAGCCGACATGTTAAGTAAGGGATTACTGGGTGGTGAAGCATGGAGCTCATGGC ACAAAGAGGATTCTATCCATGTGGGTGTTCGTTGTATTGAGATGTTGATCGAATCCACTGGCATGGTTTCTCTGCATCGTCAAAACGCGGGTGTTGTAGGACAAGACTCGGAAACAATTGAA CTGGCGCCTGAGTACGCTGAGGCGATCGCGACACGCGCAGGAGCCTTGGCTGGCATTTCCCCTATGTTTCAGCCTTGTGTAGTACCACCTAAACCATGGACTGGGATTACCGGGGGAGGT TATTGGGCTAACGGTCGCCGCCCCTTGGCTCTGGTGCGCACGCACTCGAAAAAAGCCCTTATGCGTTATGAAGACGTTTACATGCCAGAAGTCTATAAGGCTATCAATATTGCTCAAAACAC GGCTTGGAAGATTAACAAAAAAGTATTGGCGGTGGCCAATGTTATTACCAAGTGGAAACATTGCCCTGTTGAAGATATCCCAGCCATCGAACGCGAAGAATTACCTATGAAGCCGGAAGATA TTGATATGAACCCAGAGGCATTGACTGCTTGGAAACGTGCCGCCGCCGCGGTCTATCGTAAGGACAAAGCACGTAAGTCGCGTCGTATCTCATTGGAATTTATGCTTGAACAGGCAAACAA GTTCGCTAATCACAAGGCTATCTGGTTCCCATATAATATGGATTGGCGCGGCCGTGTGTACGCGGTCAGCATGTTCAACCCTCAAGGTAATGACATGACAAAGGGACTGCTGACCCTGGCA AAAGGAAAACCGATTGGAAAAGAGGGGTACTATTGGTTGAAGATCCATGGAGCCAATTGCGCCGGCGTCGACAAGGTGCCGTTTCCCGAACGCATTAAATTTATTGAGGAGAACCATGAAA ACATCATGGCTTGTGCTAAATCGCCATTAGAAAATACGTGGTGGGCGGAGCAGGATTCGCCTTTCTGTTTTTTAGCGTTCTGCTTTGAATACGCAGGCGTTCAACACCATGGGCTGTCTTACA ATTGTTCACTTCCTCTTGCTTTTGATGGAAGTTGTTCCGGGATCCAGCACTTTAGTGCGATGCTGCGCGACGAGGTCGGGGGCCGTGCGGTGAATTTGTTACCAAGCGAAACTGTGCAGGA CATCTACGGAATTGTGGCCAAAAAAGTCAACGAAATCTTGCAAGCAGATGCCATTAATGGGACTGACAACGAAGTCGTGACTGTGACTGACGAGAACACTGGCGAAATTTCTGAGAAGGTTA AACTGGGGACTAAGGCCCTTGCCGGCCAATGGTTGGCCTATGGCGTGACACGCTCAGTAACTAAACGTAGCGTTATGACCTTAGCTTATGGAAGTAAAGAATTTGGATTCCGCCAACAGGT	*Enterobacteria Phage T7*	pT7-911	Amp	E. coli BL21-Gold (DE3)

	GCTGGAGGACACTATTCAACCTGCGATCGATTCAGGTAAGGGACTGATGTTCACCCAGCCTAATCAAGCAGCGGGTTATATGGCTAAATTAATTTGGGAATCCGTCTCAGTAACTGTGGTCG CTGCCGTGGAAGCGATGAACTGGTTAAAATCCGCGGCGAAGTTGTTGGCCGCAGAAGTGAAGGACAAGAAAACAGGAGAAATCCTGCGTAAGCGCTGCGCTGTCCATTGGGTCACCCCGG ACGGATTTCCGGTTTGGCAGGAGTATAAAAAACCGATTCAGACCCGCCTTAATTTAATGTTTTTGGGTCAATTCCGTCTGCAACCGACGATCAATACTAATAAAGATAGCGAAATCGACGCGC ACAAGCAGGAGTCGGGGATTGCTCCTAACTTCGTGCACAGCCAGGATGGATCGCACCTGCGCAAAACAGTGGTTTGGGCCCACGAAAAATACGGGATCGAAAGTTTTGCGCTGATCCACG ACAGTTTCGGTACCATCCCGGCTGATGCTGCCAACCTGTTTAAAGCCGTCCGCGAAACTATGGTTGACACGTACGAAAGTTGCGACGTGCTTGCAGACTTTTATGATCAATTCGCTGACCAA TTACACGAAAGTCAGTTGGATAAAATGCCTGCGTTGCCCGCGAAGGGGAACTTGAACCTGCGTGATATCCTTGAGTCAGATTTTGCATTCGCTTAATAATACTAGAGCCAGGCATCAAATAAA ACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGC GGCCGCTGCAG -3’				

Chlor = Chloramphenicol, Kan = Kanamycin, Amp = Ampicillin

Table A2-7: Reporter constructs

Protein	DNA Sequence	Vector	Antibiotic	Strain
EYFP: Spinach	5’- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAGAAAGAGGAGAAATACTAGATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGG TCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTG CCCTGGCCCACCCTCGTGACCACCTTCGGCTACGGCCTGCAATGCTTCGCCCGCTACCCCGACCACATGAAGCTGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAG CGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAA CATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAG CGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGC GCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGGGTGGTAGCGGTGGTGGTAGCGGTAAAGACTATAAGGATGACGATGAC AAATAATAATACTAGAGGATGTAAGTGAATGAAATGGTGAAGGACGGGTCCAGTAGGCTGCTTCGGCAGCCTACTTGTTGAGTAGAGTGTGAGCTCCGTAACTACTTACATCTACTAGAGCC AGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGT TTATATACTAGTAGCGGCCGCTGCAG -3’	pJET	Amp	E. coli BL21-Gold (DE3)

Table A2-8: TX-TL protein purity characterization

TX-TL Protein	Molecular Weight (kDa)	Molar Extinction Coefficient at A_{280nm} ($M^{-1}cm^{-1}$)	Concentration (μM)	Concentration in Reaction (μM)	Protein Purity	A_{260}/A_{280}
AlaRS	97.4	71195	131	0.72	85.0% ± 4.2%	1.20
ArgRS	66.0	62020	24	0.03	92.4% ± 2.8%	0.77
AsnRS	53.9	62590	250	0.41	99.4% ± 0.2%	0.66
AspRS	69.4	43110	141	0.12	92.9% ± 1.4%	0.64
CysRS	53.5	62590	72	0.02	98.2% ± 0.7%	0.56
GlnRS	64.9	77405	30	0.06	93.7% ± 0.3%	0.60
GluRS	55.2	71195	226	0.24	97.6% ± 0.6%	0.61
GlyRSα	36.1	60070	60	0.28	71.4% ± 1.3%	0.79
GlyRSβ	78.2	57995	48	0.13	95.5% ± 1.0%	0.53
HisRS	48.4	55600	58	0.02	94.5% ±3.1%	0.54
IleRS	105.6	176545	72	0.38	93.7% ± 0.9%	0.57
LeuRS	98.6	169765	36	0.04	90.1% ± 1.7%	0.62
LysRS	58.9	31860	190	0.10	96.2% ± 0.2%	0.77
MetRS	76.3	96760	16	0.03	72.2% ± 0.4%	0.84
PheRS**α**	38.2	21430	32	0.45	84.2 % ± 2.0%	0.79
PheRS**β**	88.7	65610	65	0.19	89.1% ± 0.9%	0.62
ProRS	65.0	54320	54	0.15	88.8% ± 1.1%	0.71
SerRS	49.8	34630	103	0.04	95.93% ± 0.57%	0.56
ThrRS	75.4	97010	33	0.08	94.1% ± 1.1%	0.63
TrpRS	38.8	21430	64	0.15	99.32% ± 0.11%	0.56
TyrRS	48.9	49765	33	0.02	89.9% ± 0.9%	0.94
ValRS	109.5	171240	21	0.02	95.3% ± 1.0%	0.60
IF1	9.7	2980	140	1.03	98.8% ± 0.4%	N/A
IF2	98.7	27515	55	0.41	95.3% ± 0.6%	0.91
IF3	21.9	4470	15	0.46	91.8% ± 5.4%	N/A
EF-G	78.9	61435	142	0.63	99.9% ± 0.1%	0.58
EF-Tu	44.7	20525	93	11.19	99.7% ± 0.0%	0.90
EF-Ts	31.8	4595**	156	1.57	99.0% ± 0.2%	N/A
RF1	41.9	21555	140	0.24	99.9% ± 0.0%	1.07
RF2	42.6	44015	95	0.23	72.1% ± 2.0%	0.64
RF3	60.9	41745	106	0.16	99.0% ± 0.5%	0.83
MTF	35.5	44585	135	0.56	92.6% ± 1.0%	0.56
RRF	22.0	2980	90	0.46	98.5% ± 1.2%	N/A
MK	25.0	10430	105	0.80	95.8% ± 1.4%	N/A
*CK	44.5	37150	N/A	0.09	N/A	N/A
NDK	18.7	4470	16	0.16	47.2% ± 4.9%	N/A
PPiase	22.2	6335	95	0.05	93.6% ± 6.7%	N/A
T7 RNAP	100.2	141010	20	0.10	97.8% ± 0.2%	0.86

*CK was purchased from Roche (details included in cost analysis)
**Concentration was determined using the molar extinction coefficient at A_{210nm} (1193000 $M^{-1}cm^{-1}$) (*286*)
Proteins without tryptophan do not provide reliable A_{280} readings, therefore N/A is given for those A_{280nm}/A_{260nm} ratios.

Table A2-9: TX-TL protein expression and purification observations

TX-TL Protein	Molecular Weight (kDa)	Expression Levels	Ni^{2+} NTA Observed Contamination	SEC Observed Peaks	mg Purified
AlaRS	97.4	Medium	Yes	2 contaminating peaks (one shoulder)	19.43
ArgRS	66.0	High	Yes (single band)	Contaminating peak, good separation	0.37
AsnRS	53.9	High	Yes (faint bands)	Small contaminating peak, good separation	26.83
AspRS	69.4	High	No	Single peak	4.58
CysRS	53.5	High	No	N/A	4.28
GlnRS	64.9	Medium	Yes (faint bands)	Small contaminating peak, good separation	0.81
GluRS	55.2	Medium	Yes (faint bands)	Small contaminating peak, good separation	5.52
GlyRSα	36.1	High	Yes (faint bands)	Contaminating peak, poor separation	1.44
GlyRSβ	78.2	Medium	No	Small contaminating peak, poor separation	6.56
HisRS	48.4	Medium	No	Slight shoulder	7.84
IleRS	105.6	High	Yes (faint bands)	Contaminating peak, poor separation	1.53
LeuRS	98.6	Low	Yes (faint bands)	2 contaminating peaks, poor separation	0.20
LysRS	58.9	High	Yes (faint bands)	Contaminating peak, poor separation	3.93
MetRS	76.3	Low	Yes (faint bands)	Contaminating peak, poor separation	0.50
PheRS**α**	38.2	High	Yes (single band)	Small contaminating peak, good separation	0.71
PheRS**β**	88.7	Low	No	Small contaminating peak, good separation	2.62
ProRS	65.0	Low	Yes (faint bands)	Contaminating peak, poor separation	2.05
SerRS	49.8	High	Yes (single band)	Slight shoulder	4.18
ThrRS	75.4	Medium	Yes (faint bands)	Slight shoulder	2.41
TrpRS	38.8	High	No	Large shoulder	2.44
TyrRS	48.9	High	Yes (faint bands)	Large shoulder	3.78
ValRS	109.5	High	Yes (faint bands)	Small contaminating peak, good separation	1.20
IF1	9.7	Low	Yes (faint bands)	No tryptophan, low A280 value	4.79
IF2	98.7	Low	Yes (faint bands)	Multiple shoulders	1.02
IF3	21.9	Low	Yes (faint bands)	No tryptophan, low A280 value	1.33
EF-G	78.9	High	Yes (faint bands)	Contaminating peak, good separation	0.16
EF-Tu	44.7	High	Yes (faint bands)	Contaminating peak, good separation	67.24
EF-Ts	31.8	High	Yes (faint bands)	No tryptophan, low A280 value	4.16
RF1	41.9	High	No	N/A	2.48
RF2	42.6	Medium	Yes (faint bands)	Small contaminating peak, good separation	19.46
RF3	60.9	High	No	Small contaminating peak, good separation	4.65
MTF	35.5	Medium	Yes (faint bands)	Small contaminating peak, good separation	4.87
RRF	22.0	Medium	Yes (faint bands)	No tryptophan, low A280 value	3.96
MK	25.0	Medium	Yes (faint bands)	No tryptophan, low A280 value	1.84
CK	44.5	N/A	N/A	N/A	100
NDK	18.7	Low	Yes (faint bands)	No tryptophan, low A280 value	0.90
PPiase	22.2	Low	Yes (faint bands)	No tryptophan, low A280 value	1.05
T7 RNAP	100.2	High	No	Single peak	20.04

*For protein expression, observed band intensity was classified as high (clear increase in band intensity), medium (observable increase in band intensity), and low (difficult to distinguish from leaky expression) relative to a non-induced sample.

Table A2-10: Contaminants in individually purified TX-TL proteins by mass spectrometry

Gene	Protein	Samples
ahpF	Alkyl hydroperoxide reductase subunit F	ArgRS
alaS	Alanine tRNA ligase	IleRS
argS	Arginine tRNA ligase	AlaRS
asnS	Asparagine tRNA ligase	ArgRS, IF2
bcsA	Cellulose synthase catalytic subunit [UDP-forming]	AlaRS
can	Carbonic anhydrase 2	LeuRS
crp	cAMP-activated global transcriptional regulator CRP	GlyRS α, PheRS α, MTF, RF2, GlnRS
dnaK	Chaperone protein DnaK	MetRS, ThrRS, AlaRS
folE	GTP cyclohydrolase 1	PheRSα, RF2, GlyRSα
fur	Ferric uptake regulation protein	GlyRSα
fusA	Elongation factor G	IF3
glmS	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	LeuRS, MetRS, PheRSb, ProRS, ValRS, ThrRS
hfq	RNA-binding protein Hfq	MetRS
lpxA	Acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine O-acyltransferase	ProRS, ArgRS, LeuRS
lysS	Lysine tRNA ligase	SerRS
lysU	Lysine--tRNA ligase, heat inducible	LysRS
murE	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase	GlyRSα
nanK	N-acetylmannosamine kinase	GlyRSα
pheA	Bifunctional chorismate mutase/prephenate dehydratase	MetRS
prfC	Release factor 3	PheRSα
purU	Formyltetrahydrofolate deformylase	MetRS, PheRSβ
rpiA	Ribose-5-phosphate isomerase A	AspRS
rplX	50S ribosomal protein L24	TyRS
rsuA	Ribosomal small subunit pseudouridine synthase A	IF3
slyD	FKBP-type peptidyl-prolyl cis-trans isomerase SlyD	GlyRSα, MetRS, RF2, MK
sthA	Soluble pyridine nucleotide transhydrogenase	RF2
sucA	2-oxoglutarate dehydrogenase E1 component	PheRSβ
tsf	Elongation factor Ts	HisRS
tufB	Elongation factor Tu 2	MK
tyrS	Tyrosine tRNA ligase	T7 RNAP
ydcF	Protein YdcF	IF3
ymiA	Protein YmiA	AlaRS

*Relative abundance $\geq$ 1% of the sample
*Purple = common Ni^{2+}NTA contaminant
*Blue = Translation component (TX-TL or ribosomal protein)

Table A2-11: In-house energy solution cost estimate

Component	Catalog Number	Company	Amount	Bulk Cost (CAD)	Amount/25 µL reaction	Cost/25 µL reaction (CAD)	Cost/1 µL reaction (CAD)
Amino Acids	LAA21-1KT	Sigma-Aldrich	1 g of each	$642.00	20.00 µg	0.0006420	0.0000257
Magnesium Acetate	M0631	Sigma-Aldrich	100 g	$29.70	63.00 µg	0.0000187	0.0000007
Potassium Glutamate	49601	Sigma-Aldrich	100 g	$70.50	508.00 µg	0.0003581	0.0000143
DTT	BP172	Fischer Scientific	25 g	$129.52	3.86 µg	0.0000200	0.0000008
ATP	A26209	Sigma-Aldrich	1 g	$59.90	27.56 µg	0.0016505	0.0000660
GTP	G8877	Sigma-Aldrich	250 mg	$370.00	26.15 µg	0.0387020	0.0015481
CTP	C1506	Sigma-Aldrich	1 g	$653.00	15.68 µg	0.0102377	0.0004095
UTP	U6750	Sigma-Aldrich	250 mg	$120.00	13.75 µg	0.0066000	0.0002640
tRNA	10109550001	Roche	8000 U A_{260}	$756.00	1.30 U A_{260}	0.1228500	0.0049140
Creatine Phosphate	27920	Sigma-Aldrich	1 g	$117.00	164.00 µg	0.0191880	0.0007675
Folinic Acid	AC230312500	Acros Organics	250 mg	$272.50	0.25 µg	0.0002725	0.0000109
Spermidine	S2626	Sigma-Aldrich	1 g	$62.10	7.30 µg	0.0004533	0.0000181
Buffer (TAK)						0.0000043	0.0000002
Lab Supplies						0.0467497	0.0018700
Work						0.1562500	0.0062500
					Total	**0.4039970**	**0.0161599**

Table A2-12: Protein purification cost estimate

Component	Catalog Number	Company	Amount	Bulk Cost (CAD)	Amount/ purification	Cost/ purification (CAD)	Notes
Ni Sepharose® 6Fast Flow	GE17-5318-02	Sigma-Aldrich	100 mL	$1,330.00	3 mL	$7.98	-Column can be regenerated and reused
Amicon Ultra-15 Centrifugal Filter Unit	UFC901096 UFC903096 UFC905096	Millipore	96 columns	$1,400.00	2 columns	$29.17	
SEC Purification						$178.75	-Resin can be reused -Based on core facility prices
LB Media + Antibiotics						$1.70	
Buffers						$12.80	
Lab Supplies						$10.64	
Work						$62.50	-4 per week
					Total	**$303.54 per purification**	

Table A2-13: Summary of Purified Protein and Cost of Factor Mix

	Protein	mg purified	Required ng/25 µL reaction	Required ng/1 µL reaction	Reactions (25 µL)/ purification	Cost/ purification (CAD)	Cost/25 µL reaction (CAD)	Cost/1 µL reaction (CAD)
1	AlaRS	19.43	1753	70	11083	$303.54	0.027387	0.001095
2	ArgRS	0.37	50	2	7440	$303.54	0.040798	0.001632
3	AsnRS	26.83	553	22	48562	$303.54	0.006251	0.000250
4	AspRS	4.58	208	8	22000	$303.54	0.013797	0.000552
5	CysRS	4.28	27	1	160000	$303.54	0.001897	0.000076
6	GlnRS	0.81	97	4	8333	$303.54	0.036425	0.001457
7	GluRS	5.52	331	13	16667	$303.54	0.018212	0.000728
8	GlyRSα	1.44	253	10	5714	$303.54	0.053119	0.002125
9	GlyRSβ	6.56	254	10	25846	$303.54	0.011744	0.000470
10	HisRS	7.84	24	1	324000	$303.54	0.000937	0.000037
11	IleRS	1.53	1004	40	1526	$303.54	0.198870	0.007955
12	LeuRS	0.20	99	4	2000	$303.54	0.151769	0.006071
13	LysRS	3.93	147	6	26640	$303.54	0.011394	0.000456
14	MetRS	0.50	57	2	8667	$303.54	0.035024	0.001401
15	PheRSα	0.71	429	17	1658	$303.54	0.183100	0.007324
16	PheRSβ	2.62	421	17	6211	$303.54	0.048875	0.001955
17	ProRS	2.05	244	10	8400	$303.54	0.036136	0.001445
18	SerRS	4.18	50	2	84000	$303.54	0.003614	0.000145
19	ThrRS	2.41	151	6	16000	$303.54	0.018971	0.000759
20	TrpRS	2.44	145	6	16800	$303.54	0.018068	0.000723
21	TyrRS	3.78	24	1	154800	$303.54	0.001961	0.000078
22	ValRS	1.20	55	2	22000	$303.54	0.013797	0.000552
23	MTF	4.79	497	20	9643	$303.54	0.031478	0.001259
24	IF1	1.02	250	10	4078	$303.54	0.074439	0.002978
25	IF2	1.33	1012	40	1317	$303.54	0.230465	0.009219
26	IF3	0.16	252	10	652	$303.54	0.465426	0.018617
27	EF-G	67.24	1243	50	54095	$303.54	0.005611	0.000224
28	EF-Tu	4.16	12499	500	332	$303.54	0.913064	0.036523
29	EF-Ts	2.48	1247	50	1987	$303.54	0.152742	0.006110
30	RF1	19.46	251	10	77500	$303.54	0.003917	0.000157
31	RF2	4.65	245	10	18957	$303.54	0.016012	0.000640
32	RF3	4.87	244	10	20000	$303.54	0.015177	0.000607
33	RRF	3.96	253	10	15652	$303.54	0.019393	0.000776
34	MK	1.84	500	20	3675	$303.54	0.082596	0.003304
35	*CK	100	100	4	999844	$219	0.000219	0.000009
36	NDK	0.90	75	3	12000	$303.54	0.025295	0.001012
37	PPiase	1.05	28	1	38000	$303.54	0.007988	0.000320

38	T7 RNAP	20.04	251	10	80000	$303.54	0.003794	0.000152
						Total Cost	2.9798/ **25 µL reaction**	0.1192/ 1 **µL reaction**

*CK was purchased from Roche (10127566001)

Table A2-14: Ribosome purification cost estimate

Component	Catalog Number	Company	Amount	Bulk Cost (CAD)	Amount/ purification	Cost/purification (CAD)
E. coli MRE600 cells	N/A	University of Georgia	2 kg	$5960.00	55 g	163.90
Buffers + Reagents						58.65
Lab Supplies						62.66
Work						550.00
					Total	**835.21**
Note: ~120000 pmoles of ribosomes per purification					**Cost/pmole**	**0.00696012**
Note: 60 pmoles per reaction					**Cost/25 µL reaction**	**0.41760708**
					Cost/1 µL reaction	**0.01670428**

Supplementary Figures

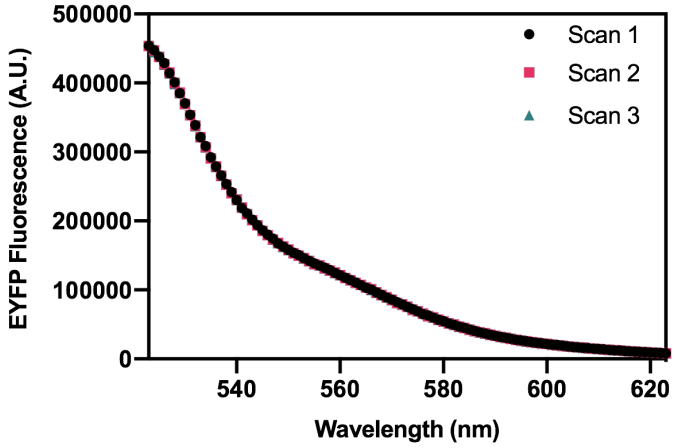

Figure A2-1: EYFP emission spectra is reproducible.

Three separate excitation and emission scans of EYFP demonstrates consistent EYFP fluorescence detected by the fluorimeter. EYFP was excited at 513 nm and emission scanned between 525-625 nm (λ_{max} = 527 nm).

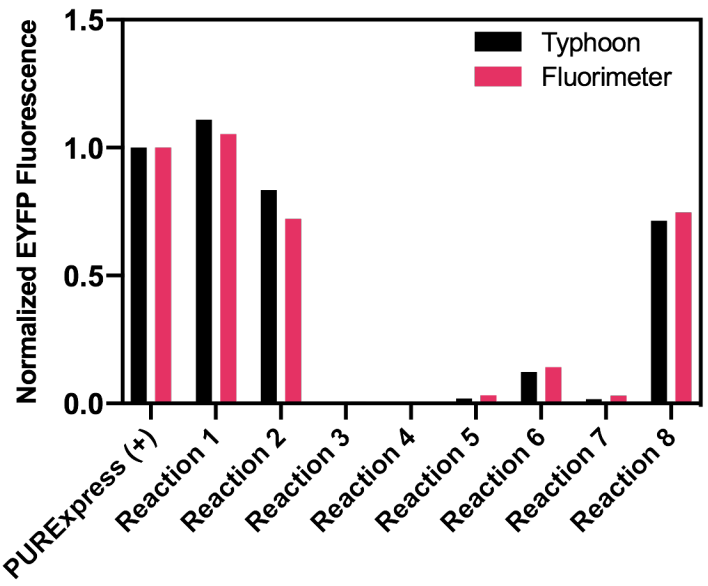

Figure A2-2: Normalized EYFP fluorescence is comparable between two different detection methods.

EYFP autofluorescence was detected using the Amersham Typhoon (excitation at 488 nm and Cy2 525BP20 emission filter) and the QuantaMaster Fluorimeter (Photon Technology International (Canada) Inc.) (excitation at 513 nm (± 1.5 nm) and emission scanned between 525-625 nm (± 2.5 nm, λ_{max} = 527 nm)) . To compare between the two methods, densitometry analysis was performed on the image obtained from the Typhoon and compared to the peak emission detected using the fluorimeter. In both approaches, the results were normalized to the control PURExpress® reaction. Overall, the results were comparable between different reaction conditions.

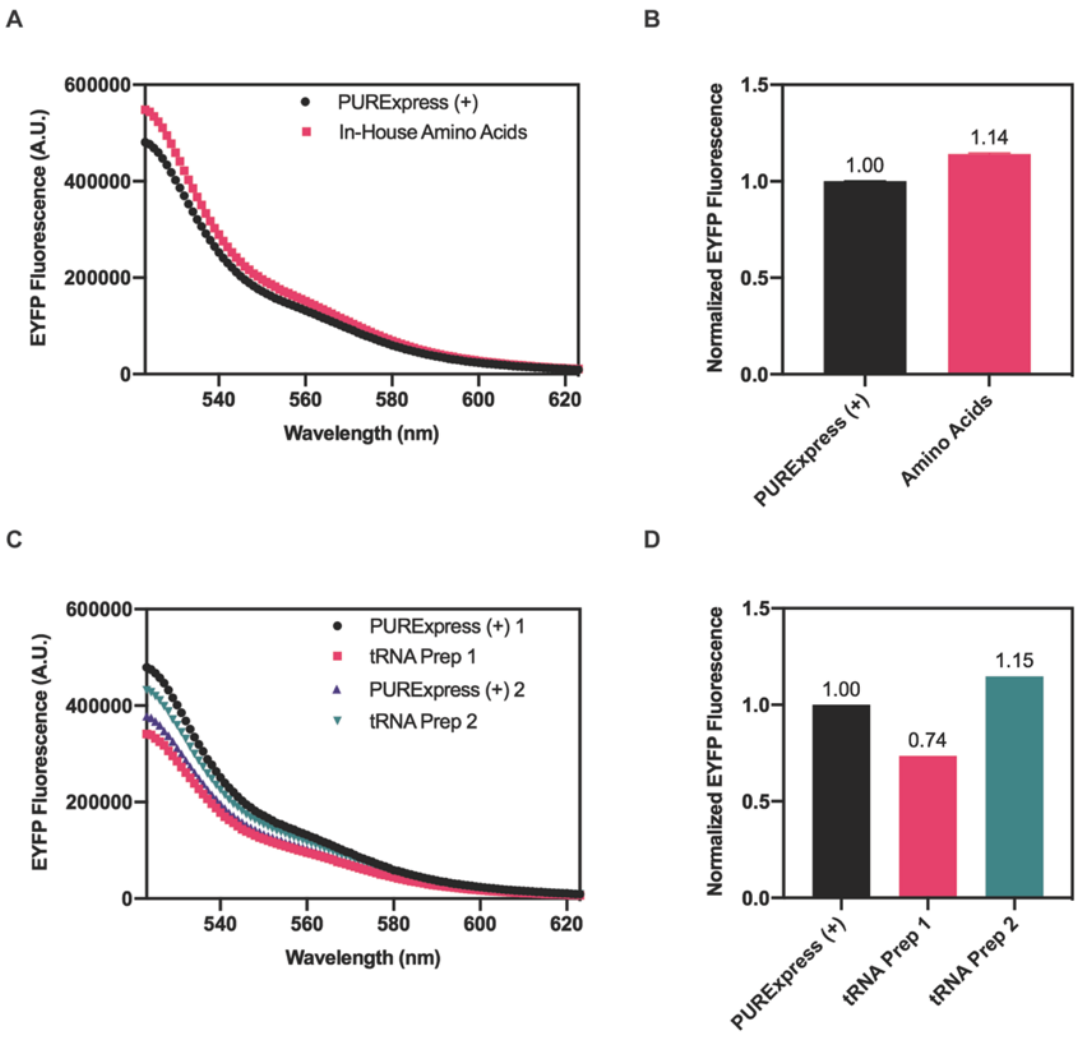

Figure A2-3: In-house prepared amino acids and tRNA are comparable to commercial components.

(A) (B) Supplementing the PURExpress® system with in-house amino acids in replacement of the commercial amino acids demonstrates translation activity comparable to the commercial system observed by similar EYFP fluorescence levels. (C) (D) Supplementing the PURExpress® system with in-house tRNA in replacement of the commercial tRNA demonstrates translation activity comparable to the commercial system observed by similar EYFP fluorescence levels. For these experiments the PURExpress® Δaa/tRNA kit was used. EYFP was excited at 513 nm and emission scanned between 525-625 nm (λ_{max} = 527 nm). In (B) and (D), fluorescence measurements were normalized to the PURExpress® system at the peak emission wavelength.

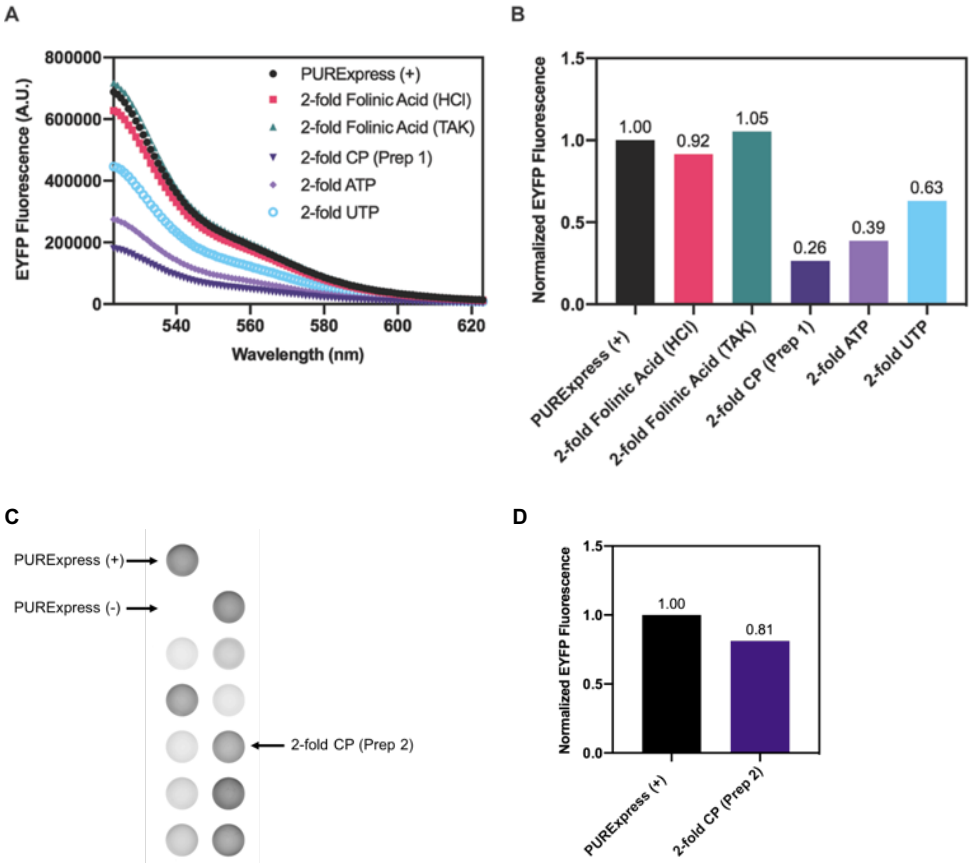

Figure A2-4: "Poisoning test" reveals potentially inhibitory components of the in-house energy solution on cell-free protein synthesis.

Individual components of the in-house energy solution were added 2-fold to the PURExpress® system (i.e. in 1-fold excess). The resulting effect on EYFP expression was measured by EYFP autofluorescence. Components that resulted in a decrease in EYFP fluorescence were investigated further, working towards the preparation of a highly functional energy solution. For example, (B) creatine phosphate (CP, prep 1) decreased EYFP fluorescence by approximately 74% (C) Preparing a fresh stock of creatine phosphate (prep 2) resulted in an improvement, decreasing EYFP fluorescence by 19%. (A) (B) EYFP fluorescence was measured using the fluorimeter. EYFP was excited at 513 nm and emission scanned between 525-625 nm (λ_{max} = 527 nm). Fluorescence measurements were normalized to the PURExpress® system at the peak emission wavelength. (C) (D) A high throughput approach was tested, utilizing a 96-well plate and imaged using the Typhoon for detecting EYFP fluorescence of multiple samples at once. Densitometry analysis was performed using ImageJ software (*206*).

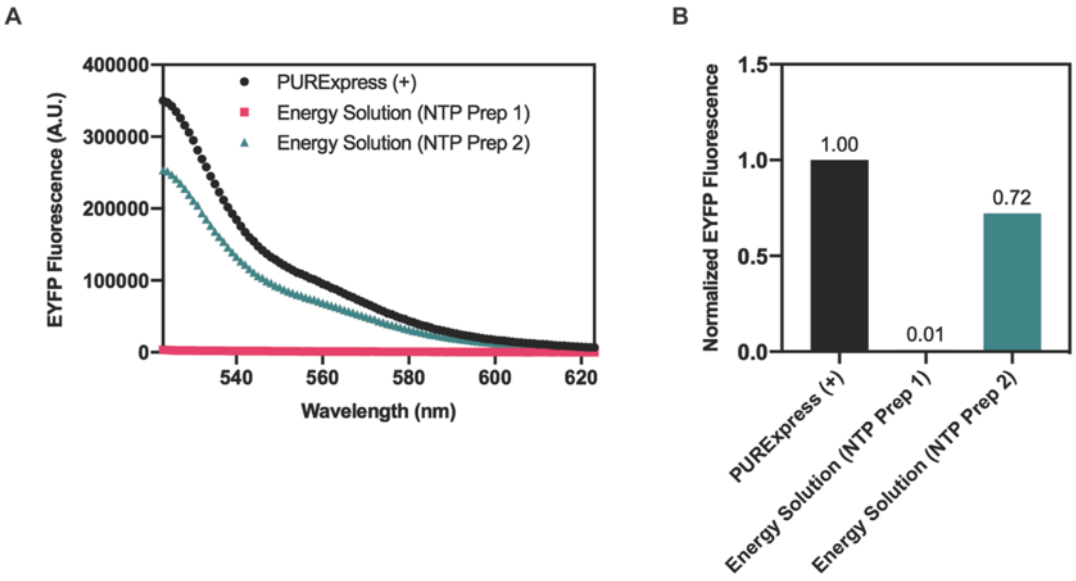

Figure A2-5: Comparison of NTP preparation on energy solution functionality.

In-house energy solution was made with NTPs prepared in two ways. Prep 1 followed methods for *in vitro* transcription reactions, dissolving ATP, CTP, and UTP in magnesium acetate, GTP in water, and setting the pH to 7.5. Prep 2 followed methods by Shimizu *et al.* (2010), dissolving each NTP in water and setting the pH to 7.0 (*210*). The in-house energy solution was combined with PURExpress® Solution B and template DNA. EYFP expression was not observed when using NTP prep 1, while 72% of the EYFP expression was observed for NTP prep 2 compared to the complete PURExpress® reaction. EYFP fluorescence was measured using the fluorimeter. EYFP was excited at 513 nm and emission scanned between 525-625 nm (λ_{max} = 527 nm). (B) Fluorescence measurements were normalized to the PURExpress® system at the peak emission wavelength.

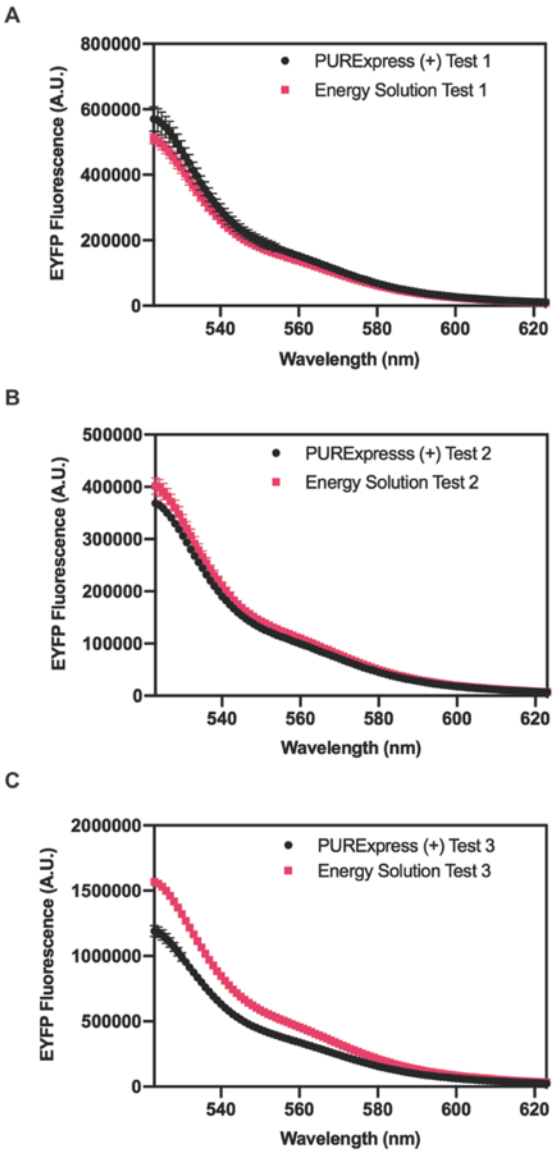

Figure A2-6: In-house energy solution is comparable to Solution A of the PURExpress® system.

The in-house energy solution was combined with PURExpress® Solution B and template DNA, and compared to the complete PURExpress® kit. Similar EYFP fluorescence levels were observed in three separate experiments (three different PURExpress kits), demonstrating a working energy solution that is comparable to the commercial equivalent (Solution A). EYFP fluorescence was measured using the fluorimeter. EYFP was excited at 513 nm and emission scanned between 525-625 nm (λ_{max} = 527 nm). (n=3)

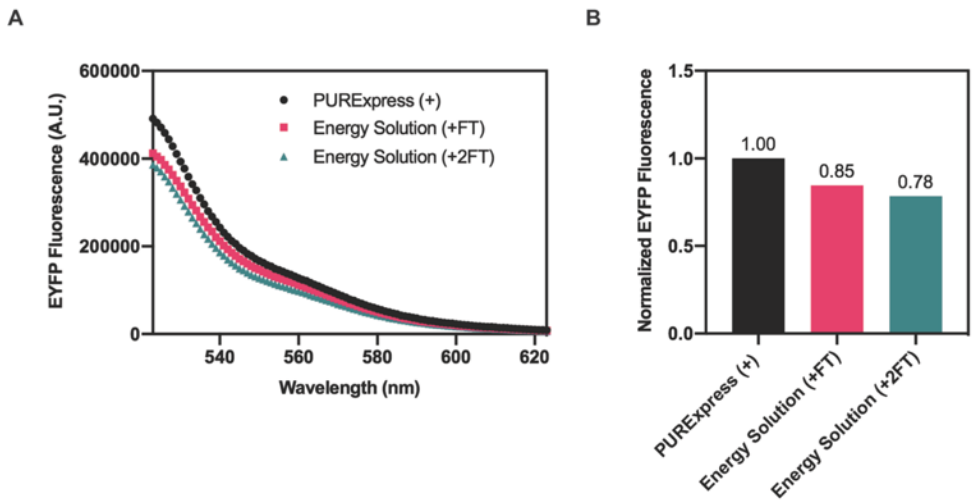

Figure A2-7: Freeze-thaw cycles decrease energy solution functionality.

Initial tests demonstrate a decrease in TX-TL activity following additional freeze-thawing observed by a decrease in EYFP fluorescence. EYFP fluorescence was measured using the fluorimeter. EYFP was excited at 513 nm and emission scanned between 525-625 nm (λ_{max} = 527 nm).

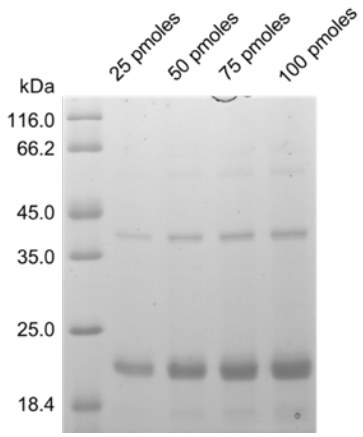

Figure A2-8: In-house purified nucleotide diphosphate kinase (NDK) protein purity.

25, 50, 75, and 100 pmoles of NDK were analyzed by 15% SDS-PAGE. The expected size of NDK is approximately 19 kDa. A contaminating band was observed at approximately 39 kDa. From the mass spectrometry data, this is assumed to be tryptophan tRNA ligase (TrpRS). Using the Image J software and performing densitometry analysis, NDK accounts for approximately 80% of the sample, which is in contrast to the mass spectrometry results which indicated NDK makes up approximately 50% of the sample.

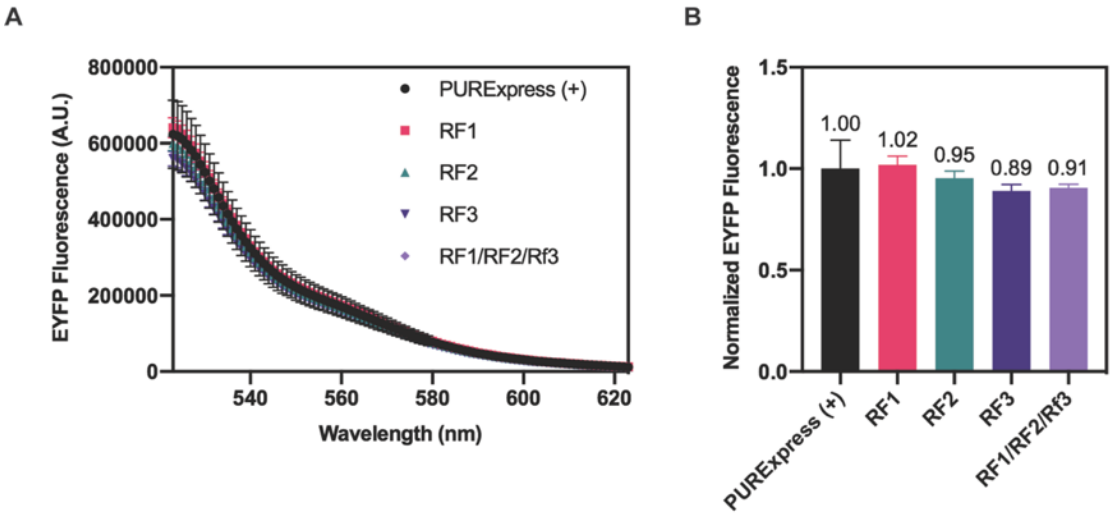

Figure A2-9: Purified release factors are comparable to commercial release factors.

In-house purified release factor were individually tested in the context of the commercial system using the PURExpress® ΔRF123. RF1, RF2, and RF3 were comparable to the commercial equivalent. EYFP fluorescence was measured using the fluorimeter. EYFP was excited at 513 nm and emission scanned between 525-625 nm (λ_{max} = 527 nm).

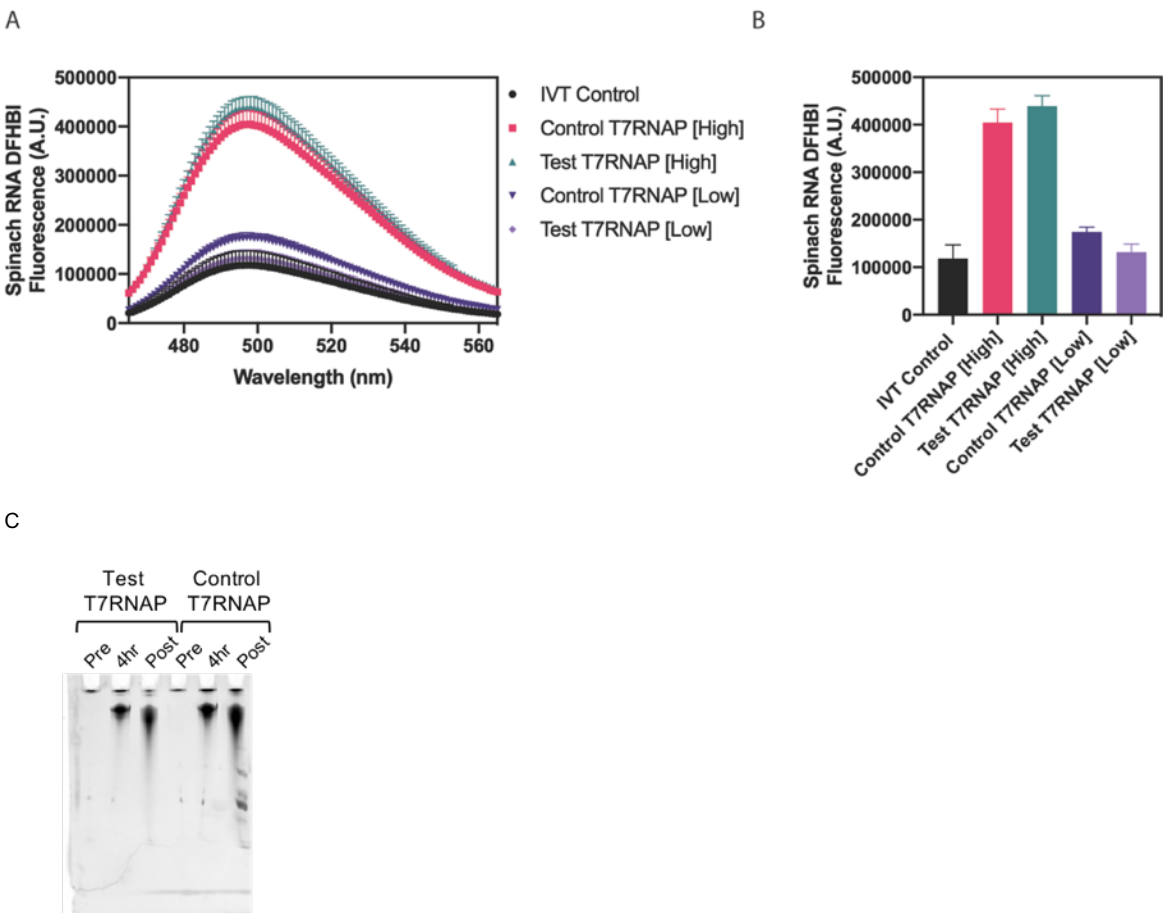

Figure A2-10: T7 RNAP successfully synthesizes RNA.

In-house purified T7 RNAP was compared to a control T7 RNAP. (A) (B) The transcription ability of in-house purified T7 RNAP was validated by measuring the fluorescence of transcribed Spinach RNA following addition of the fluorophore DFHBI. Three different reaction conditions were tested: 1) in the context of an IVT reaction, 2) in the context of a PURE reaction with 0.77 μM T7 RNAP and 0.1 μM PPiase (High), and 3) in the context of a PURE reaction with 0.1 μM T7 RNAP and 0.05 μM PPiase (Low). Spinach RNA:DFHBI was excited at 450 nm and emission scanned between 465-565nm (λ_{max} = 498 nm). (C) Urea PAGE analysis of transcribed Spinach RNA. Samples were taken before (Pre), after 4-hours (4hr), and after 16-hours (Post) of incubation at 37°C.

APPENDIX 3 – SUPPLEMENTAL MATERIAL TO CHAPTER 3

Supplemental Tables

Table A3-1: Composition of the commercial factor mix

	Protein IDs	Protein names	Gene names	MW (kDa)	Peptides	Sequence Coverage	Relative Abundance % (iBAQ)
1	P0CE48;P0CE47	Elongation factor Tu 2;Elongation factor Tu 1	tufB;tufA	43.3	34.7 ± 0.6	90.3 ± 0.6	62.761 ± 4.420
2	P0A6P1	Elongation factor Ts	tsf	30.4	25.7 ± 0.6	84.3 ± 3.8	7.292 ± 0.535
3	P0A707	Translation initiation factor IF-3;Translation initiation factor IF-3, N-terminally processed; Translation initiation factor IF-3S	infC	20.6	9.7 ± 0.6	56.9 ± 1.6	4.457 ± 0.442
4	P00957	Alanine--tRNA ligase	alaS	96.0	50.0 ± 0.0	66.9 ± 1.5	2.891 ± 0.282
5	P0A6M8	Elongation factor G	fusA	77.6	30.7 ± 0.6	60.7 ± 1.6	2.776 ± 0.581
6	P23882	Methionyl-tRNA formyltransferase	fmt	34.2	15.3 ± 1.5	55.6 ± 1.3	2.723 ± 0.142
7	P69222	Translation initiation factor IF-1	infA	8.2	3.0 ± 0.0	47.2 ± 0.0	2.489 ± 0.461
8	P0A805	Ribosome-recycling factor	frr	20.6	9.0 ± 0.0	47.6 ± 0.0	2.264 ± 0.354
9	P0A8M0	Asparagine--tRNA ligase	asnS	52.6	16.7 ± 0.6	45.1 ± 1.0	1.876 ± 0.100
10	P0A8N5;P0A8N3	Lysine--tRNA ligase, heat inducible	lysU	57.8	24.0 ± 1.0	52.4 ± 1.4	1.159 ± 0.270
11	P04805	Glutamate--tRNA ligase	gltX	53.8	16.3 ± 2.1	53.2 ± 5.9	1.090 ± 0.212
12	P0A705	Translation initiation factor IF-2	infB	97.3	37.0 ± 1.0	53.5 ± 1.6	1.060 ± 0.296
13	P00956	Isoleucine--tRNA ligase	ileS	104.3	36.0 ± 0.0	53.7 ± 0.4	0.986 ± 0.187
14	P16659	Proline--tRNA ligase	proS	63.7	24.7 ± 0.6	52.1 ± 1.5	0.920 ± 0.171
15	P07395	Phenylalanine--tRNA ligase beta subunit	pheT	87.4	30.3 ± 1.5	58.2 ± 2.0	0.776 ± 0.203
16	P0AG18	N5-carboxyaminoimidazole ribonucleotide mutase	purE	17.8	1.3 ± 0.6	10.1 ± 0.0	0.762 ± 0.240
17	P0A7I4	Peptide chain release factor 3	prfC	59.6	10.0 ± 0.0	28.5 ± 0.0	0.446 ± 0.137
18	P60906	Histidine--tRNA ligase	hisS	47.0	14.7 ± 0.6	42.8 ± 2.4	0.443 ± 0.152
19	P0A7I0	Peptide chain release factor 1	prfA	40.5	16.3 ± 0.6	51.3 ± 1.3	0.394 ± 0.102
20	P00961	Glycine--tRNA ligase beta subunit	glyS	76.8	25.0 ± 1.7	53.9 ± 3.3	0.323 ± 0.098
21	P00960	Glycine--tRNA ligase alpha subunit	glyQ	34.8	6.0 ± 0.0	29.4 ± 0.0	0.312 ± 0.156
22	P0A8L1	Serine--tRNA ligase	serS	48.4	13.0 ± 1.0	45.0 ± 1.8	0.302 ± 0.072
23	P07012	Peptide chain release factor 2	prfB	41.3	9.0 ± 0.0	26.7 ± 0.8	0.237 ± 0.060
24	P08312	Phenylalanine--tRNA ligase alpha subunit	pheS	36.8	8.0 ± 0.0	29.4 ± 1.8	0.228 ± 0.052
25	P21889	Aspartate--tRNA ligase	aspS	65.9	14.3 ± 1.5	31.6 ± 3.4	0.182 ± 0.043
26	P0AEG4	Thiol:disulfide interchange protein DsbA	dsbA	23.1	3.3 ± 0.6	20.5 ± 2.9	0.144 ± 0.047
27	P0A763	Nucleoside diphosphate kinase	ndk	15.5	2.7 ± 0.6	24.7 ± 5.7	0.103 ± 0.029
28	P00962	Glutamine--tRNA ligase	glnS	63.5	7.7 ± 0.6	23.3 ± 1.0	0.092 ± 0.031
29	P0A996	Anaerobic glycerol-3-phosphate dehydrogenase subunit C	glpC	44.1	2.0 ± 0.0	3.8 ± 0.0	0.090 ± 0.037
30	P00959	Methionine--tRNA ligase	metG	76.3	12.3 ± 1.2	26.4 ± 2.5	0.079 ± 0.026
31	P0ACJ8	cAMP-activated global transcriptional regulator CRP	crp	23.6	3.0 ± 0.0	14.8 ± 0.0	0.053 ± 0.017
32	P0A8M3	Threonine--tRNA ligase	thrS	74.0	6.3 ± 0.6	13.3 ± 0.1	0.045 ± 0.016

33	P21888	Cysteine--tRNA ligase	cysS	52.2	2.7 ± 0.6	7.1 ± 2.0	0.043 ± 0.048
34	P0AFF2	Nucleoside permease NupC	nupC	43.5	1.7 ± 0.6	10.1 ± 2.9	0.042 ± 0.014
35	P00954	Tryptophan--tRNA ligase	trpS	37.4	1.7 ± 0.6	7.6 ± 4.0	0.041 ± 0.041
36	P07813	Leucine--tRNA ligase	leuS	97.2	10.0 ± 0.0	14.5 ± 2.4	0.040 ± 0.010
37	P11875	Arginine--tRNA ligase	argS	64.7	5.0 ± 0.0	9.5 ± 0.0	0.035 ± 0.011
38	P0AGJ9	Tyrosine--tRNA ligase	tyrS	47.5	2.0 ± 0.0	6.1 ± 0.0	0.026 ± 0.013
39	P17169	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	glmS	66.9	2.0 ± 0.0	4.1 ± 0.0	0.013 ± 0.007
40	P07118	Valine--tRNA ligase	valS	108.2	3.0 ± 1.0	3.8 ± 1.4	0.009 ± 0.004

*Contaminating factors are indicated in grey

Table A3-2: Composition of the in-house factor mix.

	Protein IDs	Protein names	Gene names	MW (kDa)	Peptides	Sequence Coverage	Relative Abundance % (iBAQ)
1	P0CE48;P0CE47	Elongation factor Tu 2;Elongation factor Tu 1	tufB;tufA	43.3	30.3 ± 1.5	80.6 ± 2.6	82.557 ± 11.717
2	P0AC62	Glutaredoxin-3	grxC	9.1	2.0 ± 0.0	36.1 ± 0.0	32.549 ± 12.003
3	P0A6P1	Elongation factor Ts	tsf	30.4	26.7 ± 0.6	75.4 ± 0.7	30.858 ± 4.561
4	P0A805	Ribosome-recycling factor	frr	20.6	7.3 ± 0.6	42.7 ± 0.9	4.994 ± 2.247
5	P23882	Methionyl-tRNA formyltransferase	fmt	34.2	10.7 ± 0.6	40.1 ± 3.1	3.828 ± 0.575
6	P0A707	Translation initiation factor IF-3;Translation initiation factor IF-3, N-terminally processed;Translation initiation factor IF-3S	infC	20.6	4.7 ± 1.2	40.6 ± 6.7	3.666 ± 0.773
7	P0A8M0	Asparagine--tRNA ligase	asnS	52.6	16.3 ± 0.6	42.9 ± 1.0	2.799 ± 0.627
8	P0A6M8	Elongation factor G	fusA	77.6	19.7 ± 1.5	39.7 ± 2.4	2.571 ± 0.508
9	P00957	Alanine--tRNA ligase	alaS	96.0	25.7 ± 2.3	36.7 ± 3.5	1.888 ± 0.264
10	P00956	Isoleucine--tRNA ligase	ileS	104.3	22.0 ± 2.6	27.6 ± 4.6	1.276 ± 0.426
11	P69222	Translation initiation factor IF-1	infA	8.2	2.0 ± 0.0	38.9 ± 0.0	1.224 ± 0.092
12	P04805	Glutamate--tRNA ligase	gltX	53.8	11.3 ± 1.5	32.5 ± 6.2	1.114 ± 0.235
13	P0A763	Nucleoside diphosphate kinase	ndk	15.5	4.3 ± 0.6	37.8 ± 4.8	1.093 ± 0.317
14	P07395	Phenylalanine--tRNA ligase beta subunit	pheT	87.4	21.7 ± 1.2	39.6 ± 2.6	0.999 ± 0.121
15	P0ACJ8	cAMP-activated global transcriptional regulator CRP	crp	23.6	5.0 ± 0.0	23.8 ± 0.0	0.949 ± 0.276
16	P0A8W0	Transcriptional regulator NanR	nanR	29.5	2.0 ± 0.0	6.5 ± 0.0	0.915 ± 0.215
17	PPiase;P0A9K9	FKBP-type peptidyl-prolyl cis-trans isomerase SlyD	slyD	22.2	2.0 ± 0.0	9.5 ± 0.0	0.681 ± 0.096
18	P08312	Phenylalanine--tRNA ligase alpha subunit	pheS	36.8	6.7 ± 1.2	25.0 ± 1.9	0.666 ± 0.104
19	P00954	Tryptophan--tRNA ligase	trpS	37.4	4.3 ± 0.6	19.3 ± 3.8	0.665 ± 0.015
20	Q47689	Probable S-methylmethionine permease	mmuP	50.5	1.7 ± 0.6	2.9 ± 0.5	0.544 ± 0.482
21	P77671	Allantoinase	allB	49.6	1.3 ± 0.6	2.5 ± 1.3	0.481 ± 0.081
22	P07012	Peptide chain release factor 2	prfB	41.3	5.7 ± 1.2	18.9 ± 4.3	0.463 ± 0.137
23	P16659	Proline--tRNA ligase	proS	63.7	10.3 ± 3.5	24.4 ± 6.1	0.459 ± 0.172
24	P0A705	Translation initiation factor IF-2	infB	97.3	17.7 ± 2.1	25.2 ± 1.4	0.457 ± 0.134
25	P0A7I0	Peptide chain release factor 1	prfA	40.5	6.3 ± 0.6	18.5 ± 1.3	0.408 ± 0.106
26	P0A8N3;P0A8N5	Lysine--tRNA ligase;Lysine--tRNA ligase, heat inducible	lysS;lysU	57.6	5.7 ± 0.6	15.1 ± 0.9	0.290 ± 0.075

27	P0A7I4	Peptide chain release factor 3	prfC	59.6	3.7 ± 1.2	11.7 ± 4.6	0.289 ± 0.080
28	P00960	Glycine--tRNA ligase alpha subunit	glyQ	34.8	2.0 ± 0.0	6.9 ± 0.0	0.218 ± 0.013
29	P21889	Aspartate--tRNA ligase	aspS	65.9	3.7 ± 1.5	7.5 ± 4.4	0.073 ± 0.028
30	P0A858	Triosephosphate isomerase	tpiA	27.0	1.7 ± 0.6	6.6 ± 2.7	0.059 ± 0.011
31	P0A6T5	GTP cyclohydrolase 1	folE	24.8	1.0 ± 1.0	3.3 ± 3.4	0.052 ± 0.055
32	P17169	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	glmS	66.9	2.0 ± 1.0	4.4 ± 2.8	0.029 ± 0.025
33	P0A6Y8	Chaperone protein DnaK	dnaK	69.1	1.3 ± 0.6	2.4 ± 1.2	0.024 ± 0.009
34	P22188	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase	murE	53.3	1.0 ± 1.7	2.7 ± 4.7	0.023 ± 0.039
35	P07813	Leucine--tRNA ligase	leuS	97.2	2.0 ± 0.0	2.7 ± 0.0	0.019 ± 0.008
36	P60906	Histidine--tRNA ligase	hisS	47.0	1.3 ± 0.6	4.2 ± 1.9	0.017 ± 0.016
37	P00961	Glycine--tRNA ligase beta subunit	glyS	76.8	2.0 ± 1.0	3.0 ± 1.7	0.012 ± 0.010
38	P11875	Arginine--tRNA ligase	argS	64.7	2.0 ± 0.0	4.2 ± 0.0	0.012 ± 0.005
39	P07118	Valine--tRNA ligase	valS	108.2	1.7 ± 1.5	2.1 ± 1.9	0.011 ± 0.011
40	P0A8M3	Threonine--tRNA ligase	thrS	74.0	2.7 ± 2.9	5.5 ± 6.2	0.009 ± 0.011
41	P00962	Glutamine--tRNA ligase	glnS	63.5	0.7 ± 1.2	1.9 ± 3.2	0.007 ± 0.012

*Contaminating factors are indicated in grey

Table A3-3: Non-ribosomal proteins detected in the commercial ribosomes.

	Protein IDs	Protein names	Gene names	MW (kDa)	Peptides	Sequence Coverage (%)	Relative Abundance (%)
1	P0A832	SsrA-binding protein	smpB	18.3	9.7 ± 0.6	66.9 ± 0.0	9.11E-02 ± 8.04E-05
2	P0AFX0	Ribosome hibernation promoting factor	hpf	10.8	4.0 ± 0.0	71.6 ± 0.0	8.93E-02 ± 8.66E-05
3	P0A850	Trigger factor	tig	48.2	30.0 ± 1.0	66.8 ± 1.1	5.15E-02 ± 4.84E-06
4	P0A9B2	Glyceraldehyde-3-phosphate dehydrogenase A	gapA	35.5	22.0 ± 0.0	77.6 ± 0.0	4.88E-02 ± 4.55E-05
5	P0AAT6	Ribosomal silencing factor RsfS	rsfS	11.6	7.0 ± 0.0	53.3 ± 0.0	4.61E-02 ± 2.80E-05
6	P0AGK4	RNA-binding protein YhbY	yhbY	10.8	4.0 ± 0.0	40.2 ± 0.0	4.42E-02 ± 2.16E-05
7	P0A6F5	60 kDa chaperonin	groL	57.3	32.3 ± 2.1	72.6 ± 2.8	3.88E-02 ± 2.85E-05
8	P0A707	Translation initiation factor IF-3;Translation initiation factor IF-3, N-terminally processed;Translation initiation factor IF-3S	infC	20.6	9.0 ± 0.0	53.3 ± 0.0	3.65E-02 ± 2.44E-05
9	P0CE48;P0CE47	Elongation factor Tu 2;Elongation factor Tu 1	tufB;tufA	43.3	16.7 ± 0.6	62.9 ± 3.9	3.40E-02 ± 1.56E-05
10	P00579	RNA polymerase sigma factor RpoD	rpoD	70.3	1.3 ± 0.6	3.2 ± 0.6	3.18E-02 ± 5.50E-04
11	P61714	6,7-dimethyl-8-ribityllumazine synthase	ribE	16.2	10.0 ± 0.0	92.3 ± 0.0	2.87E-02 ± 1.67E-05
12	P0A7G2	Ribosome-binding factor A	rbfA	15.2	6.0 ± 0.0	56.4 ± 0.0	2.28E-02 ± 1.44E-05
13	P38521	Uncharacterized protein YggL	yggL	12.9	7.7 ± 1.2	73.1 ± 6.5	2.25E-02 ± 3.10E-05
14	P0A6X3	RNA-binding protein Hfq	hfq	11.2	8.3 ± 0.6	65.7 ± 20.4	2.05E-02 ± 1.84E-05
15	P07012	Peptide chain release factor 2	prfB	41.3	15.7 ± 0.6	48.4 ± 1.1	1.91E-02 ± 1.54E-05
16	P0AFG8	Pyruvate dehydrogenase E1 component	aceE	99.7	36.3 ± 1.2	53.0 ± 0.7	1.86E-02 ± 1.20E-05
17	P06959	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	aceF	66.1	28.7 ± 1.5	56.3 ± 1.1	1.85E-02 ± 5.35E-06
18	P68191	Stationary-phase-induced ribosome-associated protein	sra	5.1	2.0 ± 0.0	40.0 ± 0.0	1.30E-02 ± 2.34E-05
19	P45577	RNA chaperone ProQ	proQ	25.9	9.3 ± 0.6	45.6 ± 5.0	1.09E-02 ± 9.27E-06

20	P0A6P9	Enolase	eno	45.7	13.0 ± 1.0	45.3 ± 3.8	1.07E-02 ± 2.30E-06
21	P0A9P0	Dihydrolipoyl dehydrogenase	lpdA	50.7	18.3 ± 0.6	53.8 ± 5.3	8.31E-03 ± 1.00E-05
22	P30850	Exoribonuclease 2	rnb	72.5	23.3 ± 1.5	48.4 ± 1.7	8.24E-03 ± 6.86E-06
23	P09373;P42632	Formate acetyltransferase 1	pflB	85.4	21.7 ± 1.2	41.4 ± 0.9	6.66E-03 ± 1.95E-06
24	P0A825	Serine hydroxymethyltransferase	glyA	45.3	12.7 ± 0.6	39.8 ± 2.7	6.45E-03 ± 4.53E-06
25	P0AGD3	Superoxide dismutase [Fe]	sodB	21.3	6.0 ± 0.0	50.3 ± 0.0	6.24E-03 ± 2.42E-06
26	P0A972	Cold shock-like protein CspE	cspE	7.5	3.0 ± 0.0	53.6 ± 0.0	6.15E-03 ± 1.04E-05
27	P0A858	Triosephosphate isomerase	tpiA	27.0	6.7 ± 1.2	48.3 ± 9.1	5.93E-03 ± 6.85E-06
28	P0A717	Ribose-phosphate pyrophosphokinase	prs	34.2	8.0 ± 0.0	35.2 ± 0.0	5.90E-03 ± 5.68E-06
29	P21499	Ribonuclease R	rnr	92.1	28.0 ± 1.7	44.8 ± 1.9	5.68E-03 ± 2.37E-06
30	P0AB71	Fructose-bisphosphate aldolase class 2	fbaA	39.1	12.0 ± 0.0	43.5 ± 0.0	5.65E-03 ± 7.04E-06
31	P0A6M8	Elongation factor G	fusA	77.6	21.0 ± 0.0	44.5 ± 0.6	5.54E-03 ± 3.48E-06
32	P0ABH7	Citrate synthase	gltA	48.0	12.3 ± 2.1	50.1 ± 6.8	5.53E-03 ± 7.96E-06
33	P0A998	Bacterial non-heme ferritin	ftnA	19.4	6.3 ± 0.6	59.8 ± 3.8	5.44E-03 ± 4.56E-06
34	P07813	Leucine--tRNA ligase	leuS	97.2	3.7 ± 1.2	5.4 ± 1.1	5.25E-03 ± 6.54E-06
35	P0A9Q7	Aldehyde-alcohol dehydrogenase;Alcohol dehydrogenase;Acetaldehyde dehydrogenase [acetylating];Pyruvate-formate-lyase deactivase	adhE	96.1	22.7 ± 2.3	39.6 ± 4.0	5.10E-03 ± 1.31E-05
36	P0A9C5	Glutamine synthetase	glnA	51.9	14.0 ± 1.0	46.4 ± 3.2	5.00E-03 ± 1.37E-06
37	P39099	Periplasmic pH-dependent serine endoprotease DegQ	degQ	47.2	2.0 ± 0.0	8.6 ± 0.0	4.95E-03 ± 4.25E-06
38	P0A6D7	Shikimate kinase 1	aroK	19.5	6.3 ± 0.6	42.8 ± 6.0	4.64E-03 ± 1.81E-06
39	P0A799	Phosphoglycerate kinase	pgk	41.1	16.0 ± 1.0	67.7 ± 4.0	4.26E-03 ± 3.97E-06
40	P61889	Malate dehydrogenase	mdh	32.3	11.7 ± 0.6	55.9 ± 3.2	4.14E-03 ± 5.24E-06
41	P0A9Y6	Cold shock-like protein CspC	cspC	7.4	3.0 ± 0.0	60.9 ± 0.0	3.76E-03 ± 3.30E-06
42	P08200	Isocitrate dehydrogenase [NADP]	icd	45.8	10.3 ± 0.6	34.3 ± 3.3	3.62E-03 ± 5.81E-07
43	P0ABK5	Cysteine synthase A	cysK	34.5	9.3 ± 0.6	46.5 ± 2.3	3.32E-03 ± 2.96E-06
44	P0ACF0	DNA-binding protein HU-alpha	hupA	9.5	2.7 ± 0.6	38.1 ± 8.9	3.30E-03 ± 3.45E-06
45	P0ADG7	Inosine-5-monophosphate dehydrogenase	guaB	52.0	10.0 ± 1.0	38.7 ± 6.1	3.26E-03 ± 2.62E-06
46	P0A7Z4	DNA-directed RNA polymerase subunit alpha	rpoA	36.5	10.7 ± 0.6	39.8 ± 4.1	3.12E-03 ± 3.81E-06
47	P0A6P1	Elongation factor Ts	tsf	30.4	8.3 ± 0.6	33.7 ± 3.2	3.10E-03 ± 2.21E-06
48	P0AEK4	Enoyl-[acyl-carrier-protein] reductase [NADH] FabI	fabI	27.9	4.0 ± 0.0	20.2 ± 0.0	2.95E-03 ± 4.83E-06
49	P0A6H5	ATP-dependent protease ATPase subunit HslU	hslU	49.6	12.0 ± 0.0	36.8 ± 0.0	2.93E-03 ± 1.87E-06
50	P27302;P33570	Transketolase 1	tktA	72.2	12.7 ± 1.2	30.6 ± 4.5	2.92E-03 ± 2.69E-06
51	P0AE08	Alkyl hydroperoxide reductase subunit C	ahpC	20.8	3.3 ± 0.6	30.1 ± 4.9	2.90E-03 ± 6.94E-06
52	P0A8M0	Asparagine--tRNA ligase	asnS	52.6	11.0 ± 1.0	26.1 ± 3.5	2.49E-03 ± 3.80E-06
53	P0A6F1	Carbamoyl-phosphate synthase small chain	carA	41.4	6.3 ± 1.2	28.8 ± 5.9	2.42E-03 ± 1.79E-06
54	P62707	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	gpmA	28.6	5.7 ± 1.2	32.7 ± 5.0	2.33E-03 ± 6.33E-06
55	P0AGE9	Succinyl-CoA ligase [ADP-forming] subunit alpha	sucD	29.8	6.0 ± 1.0	30.1 ± 4.8	2.21E-03 ± 1.54E-06
56	P0AD49	Ribosome-associated inhibitor A	raiA	12.8	2.7 ± 0.6	45.4 ± 8.7	2.18E-03 ± 3.65E-06
57	P32132	GTP-binding protein TypA/BipA	typA	67.4	4.3 ± 0.6	9.1 ± 0.9	2.03E-03 ± 3.21E-06

58	P0A6Y8	Chaperone protein DnaK	dnaK	69.1	14.0 ± 1.0	30.8 ± 4.5	1.90E-03 ± 2.13E-06
59	P00509	Aspartate aminotransferase	aspC	43.6	10.0 ± 1.0	36.4 ± 3.5	1.87E-03 ± 2.04E-06
60	P0AG86	Protein-export protein SecB	secB	17.3	2.3 ± 0.6	30.1 ± 13.0	1.82E-03 ± 3.61E-06
61	P68066	Autonomous glycyl radical cofactor	grcA	14.3	3.7 ± 0.6	42.0 ± 4.5	1.77E-03 ± 1.99E-06
62	P00954	Tryptophan--tRNA ligase	trpS	37.4	6.0 ± 2.6	24.1 ± 6.7	1.75E-03 ± 4.17E-06
63	P0A7D4	Adenylosuccinate synthetase	purA	47.3	9.3 ± 0.6	31.8 ± 2.1	1.64E-03 ± 8.55E-07
64	P69776	Major outer membrane lipoprotein Lpp	lpp	8.3	1.7 ± 0.6	28.2 ± 8.9	1.58E-03 ± 3.91E-06
65	P05793	Ketol-acid reductoisomerase	ilvC	54.1	9.0 ± 1.0	20.2 ± 3.2	1.56E-03 ± 7.85E-07
66	P0A870	Transaldolase B	talB	35.2	7.7 ± 0.6	27.8 ± 0.0	1.49E-03 ± 9.84E-07
67	P0A6F9	10 kDa chaperonin	groS	10.4	2.0 ± 0.0	27.8 ± 0.0	1.49E-03 ± 2.25E-06
68	P0A9D8	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	dapD	29.9	6.0 ± 1.0	31.4 ± 8.6	1.42E-03 ± 4.88E-06
69	P0A836	Succinyl-CoA ligase [ADP-forming] subunit beta	sucC	41.4	8.3 ± 0.6	29.0 ± 1.6	1.41E-03 ± 3.51E-06
70	P0AAI5	3-oxoacyl-[acyl-carrier-protein] synthase 2	fabF	43.0	4.7 ± 1.2	23.9 ± 7.4	1.41E-03 ± 4.40E-06
71	P06996;P77747;P76335	Outer membrane protein C	ompC	40.4	1.3 ± 0.6	3.3 ± 1.4	1.40E-03 ± 5.65E-07
72	P0A9M8	Phosphate acetyltransferase	pta	77.2	12.7 ± 0.6	25.3 ± 0.7	1.39E-03 ± 1.97E-06
73	P0A6A3	Acetate kinase	ackA	43.3	4.7 ± 1.2	21.7 ± 5.5	1.26E-03 ± 4.19E-06
74	P0ABT2	DNA protection during starvation protein	dps	18.7	3.3 ± 0.6	30.3 ± 4.8	1.23E-03 ± 3.09E-06
75	P0AD61	Pyruvate kinase I	pykF	50.7	8.7 ± 0.6	22.7 ± 2.7	1.17E-03 ± 4.98E-07
76	P0A953	3-oxoacyl-[acyl-carrier-protein] synthase 1	fabB	42.6	4.3 ± 0.6	18.6 ± 1.0	1.17E-03 ± 5.41E-06
77	P0A715	2-dehydro-3-deoxyphosphooctonate aldolase	kdsA	30.8	3.7 ± 0.6	25.5 ± 2.3	1.04E-03 ± 2.30E-06
78	P0ABP8	Purine nucleoside phosphorylase DeoD-type	deoD	26.0	3.0 ± 1.0	16.2 ± 7.5	1.03E-03 ± 1.41E-06
79	P0A9T0	D-3-phosphoglycerate dehydrogenase	serA	44.2	3.7 ± 0.6	13.1 ± 1.8	9.85E-04 ± 2.31E-06
80	P60390	Ribosomal RNA small subunit methyltransferase H	rsmH	34.9	1.3 ± 0.6	8.3 ± 6.1	9.58E-04 ± 1.23E-05
81	P0ACF4	DNA-binding protein HU-beta	hupB	9.2	2.0 ± 0.0	33.3 ± 0.0	9.31E-04 ± 1.19E-06
82	P0AGD7	Signal recognition particle protein	ffh	49.8	1.3 ± 0.6	4.7 ± 1.5	9.22E-04 ± 1.50E-05
83	P05055	Polyribonucleotide nucleotidyltransferase	pnp	77.1	6.7 ± 0.6	13.8 ± 0.6	9.21E-04 ± 3.04E-06
84	P0A7B8	ATP-dependent protease subunit HslV	hslV	19.1	2.0 ± 0.0	18.2 ± 0.0	9.05E-04 ± 1.25E-06
85	P0A8L1	Serine--tRNA ligase	serS	48.4	6.0 ± 1.0	28.7 ± 3.8	8.82E-04 ± 9.11E-07
86	P00968	Carbamoyl-phosphate synthase large chain	carB	117.8	14.7 ± 2.1	20.0 ± 2.4	8.73E-04 ± 9.00E-07
87	P0ABD3	Bacterioferritin	bfr	18.5	2.0 ± 0.0 ±	20.3 ± 0.0	8.66E-04 ± 1.54E-06
88	P23721	Phosphoserine aminotransferase	serC	39.8	5.3 ± 0.6 ±	20.4 ± 1.7	8.41E-04 ± 5.99E-07
89	P69441	Adenylate kinase	adk	23.6	2.7 ± 0.6 ±	17.0 ± 2.9	8.34E-04 ± 2.63E-07
90	P0A805	Ribosome-recycling factor	frr	20.6	3.3 ± 0.6	27.0 ± 5.2	7.90E-04 ± 2.10E-06
91	P00350	6-phosphogluconate dehydrogenase, decarboxylating	gnd	51.5	6.3 ± 1.2	16.4 ± 2.2	7.72E-04 ± 1.03E-06
92	P33195	Glycine dehydrogenase (decarboxylating)	gcvP	104.4	10.0 ± 2.0	19.1 ± 3.8	7.70E-04 ± 6.44E-07
93	P0ACJ0	Leucine-responsive regulatory protein	lrp	18.9	1.7 ± 0.6	12.6 ± 6.7	7.40E-04 ± 1.19E-06
94	P0AC53	Glucose-6-phosphate 1-dehydrogenase;Extracellular death factor	zwf	55.7	8.0 ± 0.0	20.4 ± 0.0	7.11E-04 ± 3.32E-07
95	P15288	Cytosol non-specific dipeptidase	pepD	52.9	3.7 ± 0.6	11.5 ± 1.8	6.95E-04 ± 1.60E-06

96	P0AEU7	Chaperone protein Skp	skp	17.7	1.3 ± 0.6	9.1 ± 2.8	6.78E-04 ± 9.91E-07
97	P0A8V2	DNA-directed RNA polymerase subunit beta	rpoB	150.6	11.3 ± 4.0	11.9 ± 4.0	6.46E-04 ± 3.12E-07
98	P14407	Fumarate hydratase class I, anaerobic	fumB	60.1	1.3 ± 0.6	5.2 ± 3.0	6.36E-04 ± 5.55E-06
99	P42641	GTPase ObgE/CgtA	obgE	43.3	4.3 ± 0.6	19.7 ± 3.5	6.36E-04 ± 1.24E-06
100	P63284	Chaperone protein ClpB	clpB	95.6	14.7 ± 0.6	26.0 ± 1.3	6.18E-04 ± 1.24E-06
101	P0AB77	2-amino-3-ketobutyrate coenzyme A ligase	kbl	43.1	4.0 ± 1.0	11.8 ± 2.3	5.99E-04 ± 3.24E-06
102	P0AA25	Thioredoxin-1	trxA	11.8	2.0 ± 0.0	19.3 ± 0.0	5.94E-04 ± 1.07E-06
103	P61949	Flavodoxin-1	fldA	19.7	2.0 ± 0.0	25.0 ± 0.0	5.84E-04 ± 3.12E-07
104	P0A9P4	Thioredoxin reductase	trxB	34.6	2.3 ± 0.6	15.1 ± 2.0	5.81E-04 ± 9.38E-07
105	P0A6T1	Glucose-6-phosphate isomerase	pgi	61.5	5.3 ± 1.2	14.0 ± 3.1	5.56E-04 ± 1.61E-06
106	P0A786	Aspartate carbamoyltransferase catalytic chain	pyrB	34.4	3.0 ± 1.0	14.8 ± 3.3	5.32E-04 ± 1.47E-06
107	P23003	tRNA/tmRNA (uracil-C(5))-methyltransferase	trmA	42.0	2.0 ± 0.0	9.0 ± 0.0	4.63E-04 ± 4.98E-07
108	P0A910	Outer membrane protein A	ompA	37.2	3.3 ± 0.6	16.3 ± 3.7	4.55E-04 ± 2.04E-06
109	P60560	GMP reductase	guaC	37.4	1.3 ± 0.6	5.8 ± 4.0	4.53E-04 ± 2.29E-06
110	P77395	Uncharacterized protein YbbN	ybbN	31.8	3.0 ± 0.0	15.4 ± 0.2	4.51E-04 ± 1.24E-06
111	P0A944	Ribosomal-protein-alanine acetyltransferase	rimI	16.6	3.0 ± 0.0	29.7 ± 0.0	4.44E-04 ± 7.26E-07
112	P00957	Alanine--tRNA ligase	alaS	96.0	7.0 ± 2.6	11.8 ± 3.4	4.30E-04 ± 1.11E-06
113	P0C0V0	Periplasmic serine endoprotease DegP	degP	49.4	2.7 ± 0.6	10.1 ± 0.8	4.22E-04 ± 1.17E-06
114	P0AGJ9	Tyrosine--tRNA ligase	tyrS	47.5	2.3 ± 0.6	8.1 ± 3.0	4.15E-04 ± 5.36E-07
115	P0A6X7	Integration host factor subunit alpha	ihfA	11.4	2.0 ± 0.0	22.2 ± 0.0	4.12E-04 ± 1.30E-07
116	P37095	Peptidase B	pepB	46.2	4.0 ± 1.0	15.4 ± 4.4	3.94E-04 ± 4.49E-07
117	P0A8X0	UPF0307 protein YjgA	yjgA	21.4	1.3 ± 0.6	10.9 ± 6.6	3.82E-04 ± 4.64E-07
118	P0AFG6	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	sucB	44.0	2.0 ± 0.0	6.9 ± 1.3	3.80E-04 ± 1.20E-06
119	P23830	CDP-diacylglycerol--serine O-phosphatidyltransferase	pssA	52.8	1.3 ± 0.6	5.4 ± 1.8	3.70E-04 ± 2.85E-07
120	P69783	Glucose-specific phosphotransferase enzyme IIA component	crr	18.3	1.3 ± 0.6	20.3 5.4	3.62E-04 ± 1.79E-06
121	P08839	Phosphoenolpyruvate-protein phosphotransferase	ptsI	63.6	2.7 ± 1.2	6.3 ± 3.5	3.53E-04 ± 1.66E-06
122	P67910	ADP-L-glycero-D-manno-heptose-6-epimerase	hldD	34.9	2.7 ± 1.2	10.0 ± 3.3	3.53E-04 ± 1.38E-06
123	P0A6L2	4-hydroxy-tetrahydrodipicolinate synthase	dapA	31.3	1.7 ± 0.6	9.7 ± 2.2	3.51E-04 ± 2.64E-06
124	P0A9P6	ATP-dependent RNA helicase DeaD	deaD	70.5	5.0 ± 1.0	12.3 ± 1.9	3.35E-04 ± 1.71E-07
125	P07913	L-threonine 3-dehydrogenase	tdh	37.2	2.3 ± 0.6	9.8 ± 2.2	3.23E-04 ± 2.94E-07
126	P0A7D7	Phosphoribosylaminoimidazole-succinocarboxamide synthase	purC	27.0	1.7 ± 0.6	6.2 ± 2.4	3.03E-04 ± 1.27E-06
127	P0AB89	Adenylosuccinate lyase	purB	51.5	2.7 ± 0.6	10.5 ± 2.3	3.03E-04 ± 7.00E-07
128	P0ABU2	Ribosome-binding ATPase YchF	ychF	39.7	2.7 ± 0.6	11.9 ± 1.4	2.58E-04 ± 1.02E-06
129	P0A9Q1	Aerobic respiration control protein ArcA	arcA	27.3	2.0 ± 0.0	16.0 ± 0.0	2.58E-04 ± 7.41E-07
130	P76536	Probable deferrochelatase/peroxidase YfeX	yfeX	33.1	2.0 ± 0.0	12.4 ± 0.0	2.56E-04 ± 9.06E-07
131	P0A8T7	DNA-directed RNA polymerase subunit beta	rpoC	155.2	9.3 ± 2.5	9.2 ± 2.2	2.55E-04 ± 1.06E-06
132	P0A7I4	Peptide chain release factor 3	prfC	59.6	2.7 ± 0.6	9.8 ± 1.7	2.28E-04 ± 7.64E-07
133	P08312	Phenylalanine--tRNA ligase alpha subunit	pheS	36.8	2.3 ± 1.2	8.0 ± 3.7	2.20E-04 ± 7.34E-07

134	P00448	Superoxide dismutase [Mn]	sodA	23.1	1.3 ± 0.6	8.5 ± 5.6	2.19E-04 ± 1.50E-06
135	P0A9W3	Energy-dependent translational throttle protein EttA	ettA	62.4	2.7 ± 0.6	5.9 ± 1.1	2.16E-04 ± 1.82E-07
136	P0A796	ATP-dependent 6-phosphofructokinase isozyme 1	pfkA	34.8	1.3 ± 0.6	4.4 ± 3.2	2.16E-04 ± 1.42E-07
137	P17169	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	glmS	66.9	3.0 ± 0.0	7.2 ± 0.5	2.11E-04 ± 9.54E-07
138	P00864	Phosphoenolpyruvate carboxylase	ppc	99.1	4.3 ± 0.6	7.5 ± 1.7	2.07E-04 ± 1.34E-06
139	P0AEQ3	Glutamine-binding periplasmic protein	glnH	27.2	2.7 ± 0.6	17.1 ± 4.0	2.04E-04 ± 4.40E-07
140	P0AC38	Aspartate ammonia-lyase	aspA	52.4	1.7 ± 0.6	3.4 ± 1.0	1.92E-04 ± 8.40E-07
141	P17117	Oxygen-insensitive NADPH nitroreductase	nfsA	26.8	2.0 ± 0.0	13.3 ± 0.0	1.84E-04 ± 5.67E-07
142	P00490	Maltodextrin phosphorylase	malP	90.5	2.3 ± 0.6	5.2 ± 1.9	1.79E-04 ± 6.52E-08
143	P68767	Cytosol aminopeptidase	pepA	54.9	3.0 ± 0.0	7.5 ± 2.9	1.79E-04 ± 7.98E-07
144	P00961	Glycine--tRNA ligase beta subunit	glyS	76.8	3.7 ± 0.6	8.0 ± 1.0	1.71E-04 ± 2.04E-07
145	P04079	GMP synthase [glutamine-hydrolyzing]	guaA	58.7	2.0 ± 0.0	6.3 ± 0.0	1.61E-04 ± 9.52E-08
146	P0ABB4	ATP synthase subunit beta	atpD	50.3	1.3 ± 1.5	4.6 ± 5.5	1.57E-04 ± 1.99E-06
147	P0A9Q9	Aspartate-semialdehyde dehydrogenase	asd	40.0	1.3 ± 0.6	6.2 ± 3.1	1.49E-04 ± 4.54E-07
148	P12758	Uridine phosphorylase	udp	27.2	1.0 ± 1.0	8.4 ± 8.7	1.46E-04 ± 1.31E-06
149	P38038	Sulfite reductase [NADPH] flavoprotein alpha-component	cysJ	66.3	2.7 ± 1.2	8.4 ± 1.8	1.38E-04 ± 5.77E-07
150	P0A6B7	Cysteine desulfurase IscS	iscS	45.1	2.3 ± 1.5	6.0 ± 4.3	1.37E-04 ± 1.40E-06
151	P0AG30	Transcription termination factor Rho	rho	47.0	2.7 ± 0.6	7.6 ± 1.4	1.31E-04 ± 4.21E-07
152	P05791	Dihydroxy-acid dehydratase	ilvD	65.5	3.0 ± 1.0	5.3 ± 2.1	1.29E-04 ± 3.82E-07
153	P0A7E5	CTP synthase	pyrG	60.4	2.7 ± 1.2	9.0 ± 6.8	1.27E-04 ± 3.64E-07
154	P46837	Protein YhgF	yhgF	85.1	3.0 ± 2.0	7.2 ± 5.7	1.16E-04 ± 5.30E-07
155	P16659	Proline--tRNA ligase	proS	63.7	1.7 ± 0.6	4.3 ± 1.5	1.10E-04 ± 1.97E-07
156	P25665	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	metE	84.7	2.0 ± 0.0	5.1 ± 0.4	1.07E-04 ± 5.34E-07
157	P36683	Aconitate hydratase B	acnB	93.5	3.3 ± 1.5	4.2 ± 2.6	1.06E-04 ± 6.61E-07
158	P15254	Phosphoribosylformylglycinamidine synthase	purL	141.4	5.3 ± 1.2	6.6 ± 2.0	9.53E-05 ± 3.11E-07
159	P0A8N5	Lysine--tRNA ligase, heat inducible	lysU	57.8	2.0 ± 1.0	5.9 ± 2.3	7.50E-05 ± 5.42E-07
160	P0A9H3	Lysine decarboxylase, inducible	cadA	81.3	1.3 ± 0.6	3.0 ± 0.5	7.46E-05 ± 1.88E-07
161	P63224	Phosphoheptose isomerase	gmhA	20.8	1.3 ± 0.6	10.6 ± 4.2	7.46E-05 ± 6.50E-07
162	P00956	Isoleucine--tRNA ligase	ileS	104.3	1.7 ± 0.6	2.2 ± 0.8	7.13E-05 ± 1.56E-07
163	P21889	Aspartate--tRNA ligase	aspS	65.9	1.7 ± 0.6	2.8 ± 1.1	6.82E-05 ± 1.75E-07
164	P13029	Catalase-peroxidase	katG	80.0	1.7 ± 0.6	3.9 ± 1.4	5.81E-05 ± 3.94E-07
165	P07395	Phenylalanine--tRNA ligase beta subunit	pheT	87.4	1.7 ± 1.2	2.9 ± 2.3	5.80E-05 ± 2.09E-07
166	P0A6Z3	Chaperone protein HtpG	htpG	71.4	1.3 ± 0.6	2.9 ± 1.2	5.70E-05 ± 1.82E-07
167	P0AFG3	2-oxoglutarate dehydrogenase E1 component	sucA	105.1	1.7 ± 0.6	1.6 ± 0.5	5.57E-05 ± 9.04E-08
168	P04805	Glutamate--tRNA ligase	gltX	53.8	1.3 ± 0.6	3.2 ± 2.0	5.38E-05 ± 9.72E-08
169	P07118	Valine--tRNA ligase	valS	108.2	2.0 ± 1.0	2.7 ± 1.1	5.27E-05 ± 2.30E-07
170	P60906	Histidine--tRNA ligase	hisS	47.0	1.0 ± 1.0	3.9 ± 4.3	4.61E-05 ± 4.52E-07
171	P15639	Bifunctional purine biosynthesis protein PurH;Phosphoribosylaminoimidazolecarboxamide formyltransferase;IMP cyclohydrolase	purH	57.3	1.0 ± 1.0	2.2 ± 2.0	4.43E-05 ± 4.09E-07

172	P0AES4	DNA gyrase subunit A	gyrA	97.0	1.3 ± 0.6	2.5 ± 1.0	4.34E-05 ± 2.61E-07
173	P22259	Phosphoenolpyruvate carboxykinase [ATP]	pckA	59.6	0.7 ± 1.2	2.0 ± 3.5	1.07E-05 ± 1.85E-07

*Unique proteins are indicated in grey.

Table A3-4: Non-ribosomal proteins detected in the in-house ribosomes.

	Protein IDs	Protein names	Gene names	MW (kDa)	Peptides	Sequence Coverage (%)	Relative Abundance (%)
1	P38521	Uncharacterized protein YggL	yggL	12.9	8.0 ± 0.0	70.4 ± 0.0	6.94E-02 ± 2.80E-02
2	P0A832	SsrA-binding protein	smpB	18.3	9.7 ± 0.6	66.9 ± 0.0	5.90E-02 ± 1.99E-03
3	P0A6X3	RNA-binding protein Hfq	hfq	11.2	8.3 ± 0.6	77.4 ± 20.4	2.85E-02 ± 1.20E-03
4	P0AGK4	RNA-binding protein YhbY	yhbY	10.8	3.3 ± 0.6	34.0 ± 5.4	1.97E-02 ± 2.37E-03
5	P0A850	Trigger factor	tig	48.2	23.3 ± 0.6	57.6 ± 1.6	1.75E-02 ± 1.99E-03
6	P0A9B2	Glyceraldehyde-3-phosphate dehydrogenase A	gapA	35.5	16.7 ± 1.2	58.9 ± 3.5	1.25E-02 ± 9.12E-04
7	P0A6F5	60 kDa chaperonin	groL	57.3	25.0 ± 1.7	61.2 ± 1.6	8.43E-03 ± 1.17E-03
8	P0AAT6	Ribosomal silencing factor RsfS	rsfS	11.6	5.7 ± 0.6	38.1 ± 0.0	8.28E-03 ± 1.46E-03
9	P0CE48;P0CE47	Elongation factor Tu 2;Elongation factor Tu 1	tufB;tufA	43.3	15.0 ± 1.0	54.4 ± 4.1	7.49E-03 ± 8.32E-04
10	P0A9Q7	Aldehyde-alcohol dehydrogenase;Alcohol dehydrogenase;Acetaldehyde dehydrogenase [acetylating];Pyruvate-formate-lyase deactivase	adhE	96.1	10.3 ± 2.3	17.0 ± 3.6	5.69E-03 ± 1.19E-03
11	P09373;P42632	Formate acetyltransferase 1	pflB	85.4	18.3 ± 1.5	36.6 ± 2.6	5.18E-03 ± 2.70E-04
12	P0A717	Ribose-phosphate pyrophosphokinase	prs	34.2	8.3 ± 0.6	36.8 ± 2.8	3.78E-03 ± 2.08E-04
13	P60390	Ribosomal RNA small subunit methyltransferase H	rsmH	34.9	1.3 ± 0.6	8.3 ± 6.1	3.40E-03 ± 7.28E-04
14	P32132	GTP-binding protein TypA/BipA	typA	67.4	2.0 ± 1.0	4.3 ± 2.5	3.35E-03 ± 1.21E-04
15	P0A6P9	Enolase	eno	45.7	10.3 ± 1.5	37.6 ± 7.8	2.42E-03 ± 3.74E-04
16	P0A998	Bacterial non-heme ferritin	ftnA	19.4	4.3 ± 0.6	50.7 ± 3.0	2.24E-03 ± 3.49E-04
17	P0A9C5	Glutamine synthetase	glnA	51.9	10.3 ± 1.5	36.6 ± 5.7	1.91E-03 ± 5.27E-05
18	P0AB89	Adenylosuccinate lyase	purB	51.5	2.0 ± 1.0	8.1 ± 5.0	1.87E-03 ± 5.07E-04
19	P0A858	Triosephosphate isomerase	tpiA	27.0	4.0 ± 1.0	24.6 ± 8.4	1.73E-03 ± 3.41E-04
20	P06996;P77747; P76335	Outer membrane protein C	ompC	40.4	3.0 ± 0.0	11.7 ± 0.0	1.43E-03 ± 4.19E-04
21	P68066	Autonomous glycyl radical cofactor	grcA	14.3	3.0 ± 1.0	36.5 ± 9.5	1.34E-03 ± 7.08E-04
22	P0A7Z4	DNA-directed RNA polymerase subunit alpha	rpoA	36.5	7.7 ± 0.6	33.1 ± 6.1	1.32E-03 ± 2.65E-04
23	P00954	Tryptophan--tRNA ligase	trpS	37.4	2.3 ± 0.6	10.7 ± 4.7	1.29E-03 ± 3.23E-04
24	P69776	Major outer membrane lipoprotein Lpp	lpp	8.3	1.3 ± 0.6	23.0 ± 8.9	1.24E-03 ± 5.87E-04
25	P0AFG8	Pyruvate dehydrogenase E1 component	aceE	99.7	15.3 ± 3.8	23.8 ± 3.9	1.03E-03 ± 1.73E-04
26	P69441	Adenylate kinase	adk	23.6	2.0 ± 0.0	13.1 ± 0.0	9.78E-04 ± 1.55E-04
27	P0A9M8	Phosphate acetyltransferase	pta	77.2	8.7 ± 1.5	17.3 ± 2.4	9.58E-04 ± 3.47E-04
28	P0A825	Serine hydroxymethyltransferase	glyA	45.3	5.0 ± 2.0	16.6 ± 6.4	9.50E-04 ± 3.70E-04
29	P76399	Multidrug resistance protein MdtC	mdtC	111.0	2.0 ± 0.0	2.9 ± 0.0	9.33E-04 ± 2.27E-04
30	P0AE08	Alkyl hydroperoxide reductase subunit C	ahpC	20.8	2.0 ± 0.0	20.3 ± 1.8	9.24E-04 ± 2.31E-04
31	P0A6M8	Elongation factor G	fusA	77.6	11.7 ± 1.2	24.9 ± 1.9	9.16E-04 ± 1.38E-04
32	P0ADG7	Inosine-5-monophosphate dehydrogenase	guaB	52.0	6.0 ± 1.0	21.9 ± 5.5	8.22E-04 ± 7.54E-05

33	P0AB71	Fructose-bisphosphate aldolase class 2	fbaA	39.1	4.7 ± 1.2	20.5 ± 4.7	7.42E-04 ± 9.31E-05
34	P0ACF0	DNA-binding protein HU-alpha	hupA	9.5	1.7 ± 0.6	24.1 ± 6.4	7.33E-04 ± 4.27E-04
35	P06959	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	aceF	66.1	10.0 ± 2.0	20.6 ± 6.7	7.23E-04 ± 1.61E-04
36	P0A7D4	Adenylosuccinate synthetase	purA	47.3	6.0 ± 0.0	20.5 ± 1.1	6.79E-04 ± 1.11E-04
37	P0A6F1	Carbamoyl-phosphate synthase small chain	carA	41.4	2.0 ± 0.0	10.5 ± 0.0	6.02E-04 ± 2.24E-04
38	P0ABK5	Cysteine synthase A	cysK	34.5	4.7 ± 0.6	20.7 ± 1.6	5.40E-04 ± 1.42E-04
39	P0A8V2	DNA-directed RNA polymerase subunit beta	rpoB	150.6	11.3 ± 2.3	11.9 ± 2.7	4.96E-04 ± 5.38E-05
40	P0A953	3-oxoacyl-[acyl-carrier-protein] synthase 1	fabB	42.6	2.0 ± 1.0	11.6 ± 5.7	4.84E-04 ± 3.49E-04
41	P61714	6,7-dimethyl-8-ribityllumazine synthase	ribE	16.2	2.3 ± 0.6	28.6 ± 10.7	4.46E-04 ± 1.55E-04
42	P23830	CDP-diacylglycerol--serine O-phosphatidyltransferase	pssA	52.8	2.0 ± 0.0	7.5 ± 0.0	4.41E-04 ± 1.60E-04
43	P21889	Aspartate--tRNA ligase	aspS	65.9	2.3 ± 0.6	4.5 ± 1.1	4.06E-04 ± 8.48E-05
44	P0AC38	Aspartate ammonia-lyase	aspA	52.4	4.0 ± 1.0	7.5 ± 1.9	4.05E-04 ± 1.01E-04
45	P0A786	Aspartate carbamoyltransferase catalytic chain	pyrB	34.4	2.0 ± 1.0	11.8 ± 5.5	3.96E-04 ± 1.18E-04
46	P46837	Protein YhgF	yhgF	85.1	5.0 ± 0.0	12.6 ± 1.3	3.86E-04 ± 9.35E-05
47	P05055	Polyribonucleotide nucleotidyltransferase	pnp	77.1	3.3 ± 0.6	7.9 ± 0.9	3.69E-04 ± 1.17E-04
48	P0A8L1	Serine--tRNA ligase	serS	48.4	2.0 ± 0.0	14.7 ± 3.9	3.45E-04 ± 1.77E-04
49	P63284	Chaperone protein ClpB	clpB	95.6	9.3 ± 1.5	17.1 ± 3.6	3.23E-04 ± 1.04E-04
50	P0AGD7	Signal recognition particle protein	ffh	49.8	1.7 ± 1.2	5.2 ± 2.4	2.80E-04 ± 3.77E-04
51	P67910	ADP-L-glycero-D-manno-heptose-6-epimerase	hldD	34.9	2.0 ± 0.0	8.1 ± 0.0	2.69E-04 ± 4.43E-05
52	P0ACF4	DNA-binding protein HU-beta	hupB	9.2	1.3 ± 0.6	21.5 ± 10.2	2.68E-04 ± 9.33E-05
53	P0A6A3	Acetate kinase	ackA	43.3	2.7 ± 0.6	11.8 ± 1.7	2.53E-04 ± 1.10E-04
54	P0A910	Outer membrane protein A	ompA	37.2	3.7 ± 1.5	14.8 ± 5.6	2.38E-04 ± 1.62E-04
55	P27302;P33570	Transketolase 1	tktA	72.2	2.7 ± 1.2	5.0 ± 1.8	2.37E-04 ± 1.22E-04
56	P0A799	Phosphoglycerate kinase	pgk	41.1	4.0 ± 2.0	16.5 ± 8.0	2.34E-04 ± 1.14E-04
57	P0A9P0	Dihydrolipoyl dehydrogenase	lpdA	50.7	3.7 ± 0.6	11.1 ± 1.6	1.69E-04 ± 5.54E-05
58	P0A6Y8	Chaperone protein DnaK	dnaK	69.1	3.3 ± 0.6	7.5 ± 2.1	1.59E-04 ± 2.45E-05
59	P0A6P1	Elongation factor Ts	tsf	30.4	1.3 ± 1.2	6.1 ± 5.3	1.52E-04 ± 1.32E-04
60	P0A9P6	ATP-dependent RNA helicase DeaD	deaD	70.5	2.7 ± 1.5	7.1 ± 3.4	1.47E-04 ± 7.91E-05
61	P0A715	2-dehydro-3-deoxyphosphooctonate aldolase	kdsA	30.8	1.7 ± 0.6	16.5 ± 5.5	1.37E-04 ± 9.06E-05
62	P61889	Malate dehydrogenase	mdh	32.3	2.3 ± 1.5	10.8 ±± 7.9	1.34E-04 ± 1.02E-04
63	P0A8T7	DNA-directed RNA polymerase subunit beta	rpoC	155.2	7.3 ± 1.2	7.7 ± 1.3	1.32E-04 ± 3.11E-05
64	P0AAI5	3-oxoacyl-[acyl-carrier-protein] synthase 2	fabF	43.0	1.3 ± 0.6	7.4 ± 3.2	1.25E-04 ± 6.86E-05
65	P76536	Probable deferrochelatase/peroxidase YfeX	yfeX	33.1	1.3 ± 0.6	8.3 ± 3.6	1.17E-04 ± 7.85E-05
66	P0ABH7	Citrate synthase	gltA	48.0	2.0 ± 1.0	9.5 ± 6.1	1.15E-04 ± 8.69E-05
67	P0ABB4	ATP synthase subunit beta	atpD	50.3	1.7 ± 0.6	5.5 ± 2.1	1.14E-04 ± 7.17E-05
68	P17169	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	glmS	66.9	2.3 ± 0.6	5.3 ± 2.0	9.63E-05 ± 2.15E-05
69	P13029	Catalase-peroxidase	katG	80.0	2.0 ± 0.0	4.5 ± 0.0	9.47E-05 ± 3.58E-05
70	P38038	Sulfite reductase [NADPH] flavoprotein alpha-component	cysJ	66.3	1.0 ± 1.0	2.8 ± 2.6	6.54E-05 ± 5.85E-05

71	P0A7E5	CTP synthase	pyrG	60.4	2.0 ± 0.0	6.2 ± 1.9	6.38E-05 ± 1.99E-05
72	P68767	Cytosol aminopeptidase	pepA	54.9	2.0 ± 0.0	5.8 ± 0.0	6.36E-05 ± 9.23E-06
73	P08200	Isocitrate dehydrogenase [NADP]	icd	45.8	1.0 ± 1.0	2.1 ± 2.1	6.08E-05 ± 6.69E-05
74	P08839	Phosphoenolpyruvate-protein phosphotransferase	ptsI	63.6	1.7 ± 0.6	3.4 ± 1.5	5.41E-05 ± 9.37E-05
75	P33195	Glycine dehydrogenase (decarboxylating)	gcvP	104.4	1.7 ± 1.2	3.3 ± 2.8	5.20E-05 ± 1.53E-05
76	P0AC53	Glucose-6-phosphate 1-dehydrogenase;Extracellular death factor	zwf	55.7	1.7 ± 0.6	3.9 ± 2.1	4.61E-05 ± 1.28E-05
77	P0AG30	Transcription termination factor Rho	rho	47.0	1.7 ± 0.6	4.9 ± 2.0	4.25E-05 ± 1.63E-05
78	P0A6T1	Glucose-6-phosphate isomerase	pgi	61.5	1.3 ± 0.6	3.9 ± 1.8	4.23E-05 ± 2.54E-05
79	P0AEK4	Enoyl-[acyl-carrier-protein] reductase [NADH] FabI	fabI	27.9	1.0 ± 1.0	6.0 ± 5.5	3.65E-05 ± 3.19E-05
80	P0A8M0	Asparagine--tRNA ligase	asnS	52.6	1.0 ± 1.0	2.4 ± 2.8	3.26E-05 ± 3.12E-05
81	P0AGD3	Superoxide dismutase [Fe]	sodB	21.3	1.0 ± 1.0	4.3 ± 4.7	3.13E-05 ± 5.42E-05
82	P21513	Ribonuclease E	rne	118.2	1.7 ± 0.6	3.2 ± 1.6	3.12E-05 ± 1.32E-05
83	P76422	Hydroxymethylpyrimidine/phosphomethylpyrimidine kinase	thiD	28.6	0.7 ± 1.2	5.0 ± 8.7	3.12E-05 ± 5.40E-05
84	P0A836	Succinyl-CoA ligase [ADP-forming] subunit beta	sucC	41.4	1.7 ± 0.6	5.5 ± 2.1	2.57E-05 ± 1.18E-05
85	P00961	Glycine--tRNA ligase beta subunit	glyS	76.8	1.0 ± 1.0	2.2 ± 2.4	2.23E-05 ± 2.47E-05
86	P00864	Phosphoenolpyruvate carboxylase	ppc	99.1	1.3 ± 1.2	2.3 ± 2.0	1.60E-05 ± 1.90E-05
87	P15254	Phosphoribosylformylglycinamidine synthase	purL	141.4	1.0 ± 1.0	2.1 ± 2.5	1.30E-05 ± 1.42E-05
88	P22259	Phosphoenolpyruvate carboxykinase [ATP]	pckA	59.6	0.7 ± 1.2	2.1 ± 3.6	4.73E-06 ± 8.19E-06
89	P21499	Ribonuclease R	rnr	92.1	1.0 ± 1.0	2.2 ± 2.3	4.55E-06 ± 7.87E-06

*Unique proteins are indicated in grey

Supplemental Figures

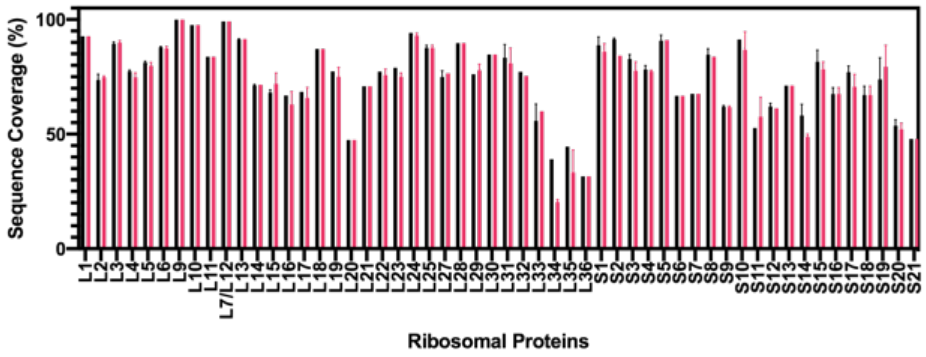

Figure A3-1: Ribosomal protein sequence coverage from LC-MS/MS.

Similar sequence coverage is detected for both samples. Black = commercial ribosomes, Pink = in-house ribosomes.

APPENDIX 4 – SUPPLEMENTAL MATERIAL TO CHAPTER 4

Supplementary Tables

Table A4-1: DNA sequences

pJET_B0034_T7_EYFP	5'- GAATTCGCGGCCGCTTCTAGAGTAATACGACTCACTATAGGGAGATACTAGAG*AAAGAGGAGAAA*TACTAG**ATG**GTGAGCAAGGGCGAGGAGCTGTT CACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACG GCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCTTCGGCTACGGCCTGCAATGCTTCGC CCGCTACCCCGACCACATGAAGCTGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGC AACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATC CTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCA CAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCT GAGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCAT GGACGAGCTGTACAAGGGTGGTAGCGGTGGTGGTAGCGGTAAAGACTATAAGGATGACGATGACAAATAATAATACTAGAGGATGTAAGTGAATGAAA TGGTGAAGGACGGGTCCAGTAGGCTGCTTCGGCAGCCTACTTGTTGAGTAGAGTGTGAGCTCCGTAACTACTTACATCTACTAGAGCCAGGCATCAAA TAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTG GGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG -3'
pSB1C3__B0034_T7_mRFP	5'- TAATACGACTCACTATAGGGAGATACTAGAG*AAAGAGGAGAAA*TACTAG**ATG**GCTTCCTCCGAAGACGTTATCAAAGAGTTCATGCGTTTCAAAGTTCG TATGGAAGGTTCCGTTAACGGTCACGAGTTCGAAATCGAAGGTGAAGGTGAAGGTCGTCCGTACGAAGGTACCCAGACCGCTAAACTGAAAGTTACCA AAGGTGGTCCGCTGCCGTTCGCTTGGGACATCCTGTCCCCGCAGTTCCAGTACGGTTCCAAAGCTTACGTTAAACACCCGGCTGACATCCCGGACTAC CTGAAACTGTCCTTCCCGGAAGGTTTCAAATGGGAACGTGTTATGAACTTCGAAGACGGTGGTGTTGTTACCGTTACCCAGGACTCCTCCCTGCAAGA CGGTGAGTTCATCTACAAAGTTAAACTGCGTGGTACCAACTTCCCGTCCGACGGTCCGGTTATGCAGAAAAAAACCATGGGTTGGGAAGCTTCCACCG AACGTATGTACCCGGAAGACGGTGCTCTGAAAGGTGAAATCAAAATGCGTCTGAAACTGAAAGACGGTGGTCACTACGACGCTGAAGTTAAAACCACC TACATGGCTAAAAAACCGGTTCAGCTGCCGGGTGCTTACAAAACCGACATCAAACTGGACATCACCTCCCACAACGAAGACTACACCATCGTTGAACA GTACGAACGTGCTGNAAGGTCGTCACTCCACCGGTGCTTAATAACGCTGATAGTGCTAGTGTAGATCGC -3'
pSB1C3_B0034_T7_eCFP	5'- TAATACGACTCACTATAGGGAGATACTAGAG*AAAGAGGAGAAA*TACTAG**ATG**GTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTG GTCGAGCTGGACGGCGACGTGAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATC TGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTGGGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAG CAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGG TGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACA ACTACATCAGCCACAACGTCTATATCACCGCCGACAAGCAGAAGAACGGCATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGT GCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAG CAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAATAA -3'
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Italicized = RBS or PSIV IRES; Bold = start codon (AUG); Underlined = secondary structure (Blue = Pseudoknot 1 (PK1), Red = PK2, Green = PK3)

Supplementary Figures

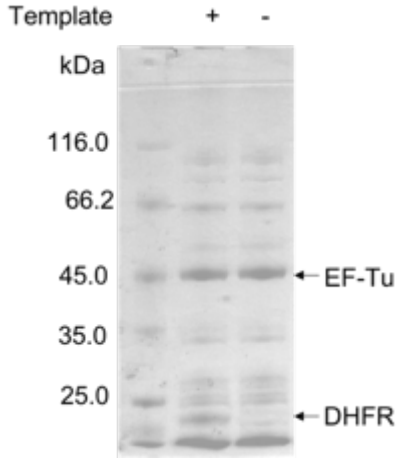

Figure A4-1: Translation efficiency of an *in vitro* reconstituted cell-free system.

15% tris-tricine analysis to detect expressed DHFR from PURExpress®. Bands corresponding to EF-Tu (~43kDa) and DHFR (~25kDa) are indicated. When template DNA is present, DHFR band intensity is less then EF-Tu band intensity (~50%) suggesting inefficient protein synthesis. Estimating the amount of DHFR from the respective band intensities in this reaction after completion indicates equimolar amounts compared to the ribosomes present in the reactions. In turn, this suggests that only a few rounds of translation occur per ribosome or that efficient translation is only initiated at a limited number of ribosomes. This can either be interpreted by only a few ribosomes performing several rounds of translation initiation and synthesis of DHFR or that the majority of the ribosomes present in the reaction only participate in one round of DHFR synthesis. Alternatively, the reaction could be limited by the availability of mRNA. In the latter case addition of S1 would not improve translation efficiency, while in the former case the increased expression of DHFR as a function of increased S1 availability supports a limitation in translation initiation. A higher band intensity of the product, DHFR, was expected if ribosomes were able to perform increased numbers of rounds of translation, and/or a large fraction of ribosomes were actively initiating translation. Densitometry analysis was performed using ImageJ software (*206*).

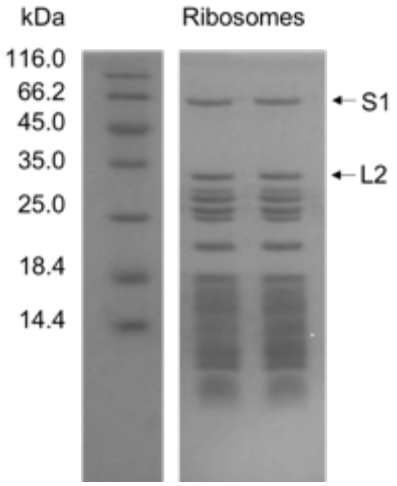

Figure A4-2: Composition of purified ribosomes used in *in vitro* TX-TL reactions.

15% SDS-PAGE analysis of ribosomal protein stoichiometry (30 pmol of ribosomes are analyzed in duplicate). Expected stoichiometry between the majority of ribosomal proteins is 1:1. The band intensity of S1 (~62kDa) is approximately 80% of the band intensity of L2 (~30kDa). For a 1:1 stoichiometry, a more intense band was expected for S1 due to its larger size. It may be concluded that S1 is present in less than stoichiometric amounts. Densitometry analysis was performed using ImageJ software(*206*).

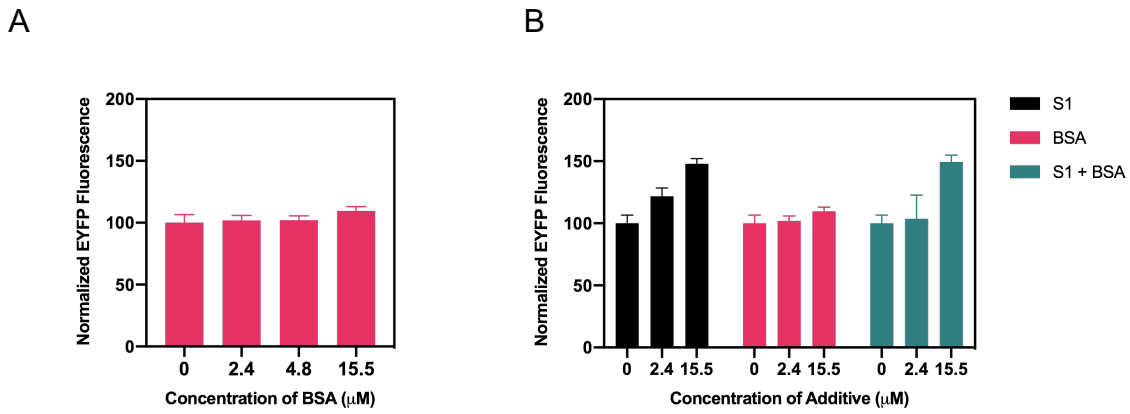

Figure A4-3: Addition of the crowding agent BSA has a minimal effect on EYFP fluorescence.

Previous studies have reported the positive effect of crowding agents, such as BSA, on cell-free protein synthesis(*74*). Due to the similar size of ribosomal protein S1 (68 kDa) and BSA (66.5 kDa), the possible effect of increased molecular bulk on cell-free protein synthesis was investigated. Overall, increasing concentrations of BSA (2.4 μM, 4.8 μM, and 15.5 μM) had a minimal effect on EYFP fluorescence, while increasing concentrations of S1 (2.4 μM and 15.5 μM) improved EYFP fluorescence by approximately 50%. These results are consistent with our hypothesis that ribosomal protein S1 aids in translation. Note that fluorescence measurements are normalized to reactions without any additives at the peak emission wavelength.

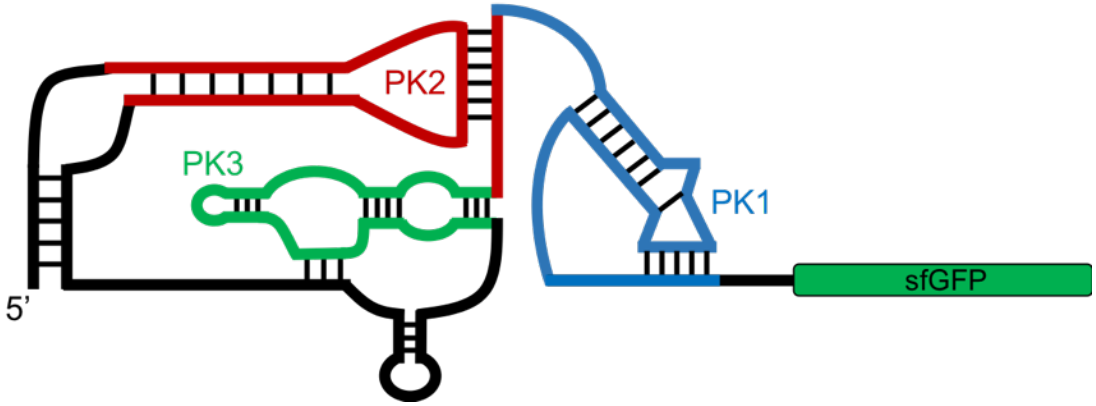

Figure A4-4: Cartoon representation of the PSIV IRES secondary structure upstream of the sfGFP coding sequence.

Pseudoknot 1 (PK1) is shown in blue; PK2 is shown in red; PK3 is shown in green. Figure adapted from Roberts and Wieden (2021) (*287*).

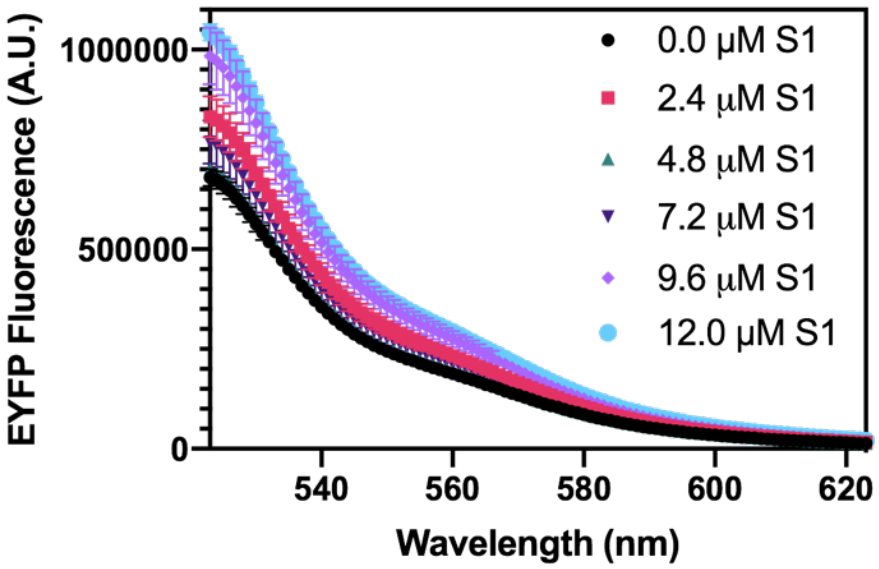

Figure A4-5: The addition of increasing concentrations of ribosomal protein S1 improves the in vitro reconstituted cell-free production of EYFP.

Ribosomal protein S1 was added in excess (2.4 μM, 4.8 μM, 7.2 μM, 9.6 μM, or 12 μM) relative to ribosome concentration (2.4 μM). EYFP was excited at 513 nm and emission scanned between 525-625 nm (λ_{max} = 527 nm). Negative control (without template) was subtracted from the emission spectra.

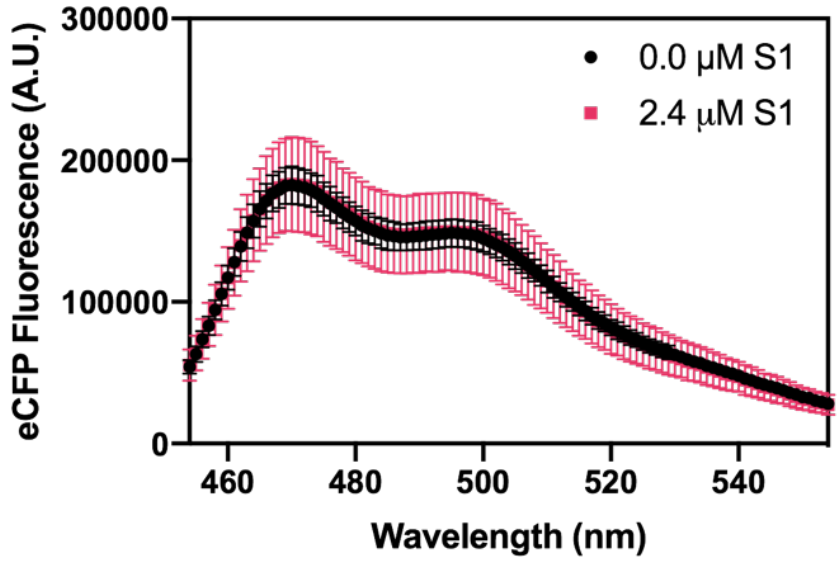

Figure A4-6: The addition of ribosomal protein S1 has no significant effect on the *in vitro* reconstituted cell-free production of eCFP.

Ribosomal protein S1 was added in 1-fold excess relative to ribosome concentration (2.4 µM). eCFP was excited at 439 nm and emission scanned between 454-554 nm (λ_{max} = 470 nm). Negative control (without template) was subtracted from the emission spectra.

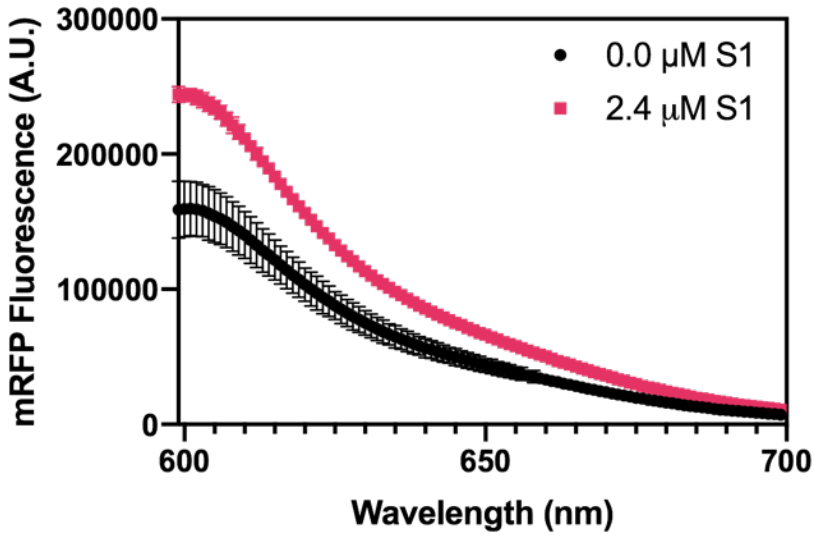

Figure A4-7: The addition of ribosomal protein S1 improve the *in vitro* reconstituted cell-free production of mRFP.

Ribosomal protein S1 was added in 1-fold excess relative to ribosome concentration (1:1). mRFP was excited at 584 nm and emission scanned between 599-699 nm (λ_{max} = 607 nm). Negative control was subtracted from the emission spectra.

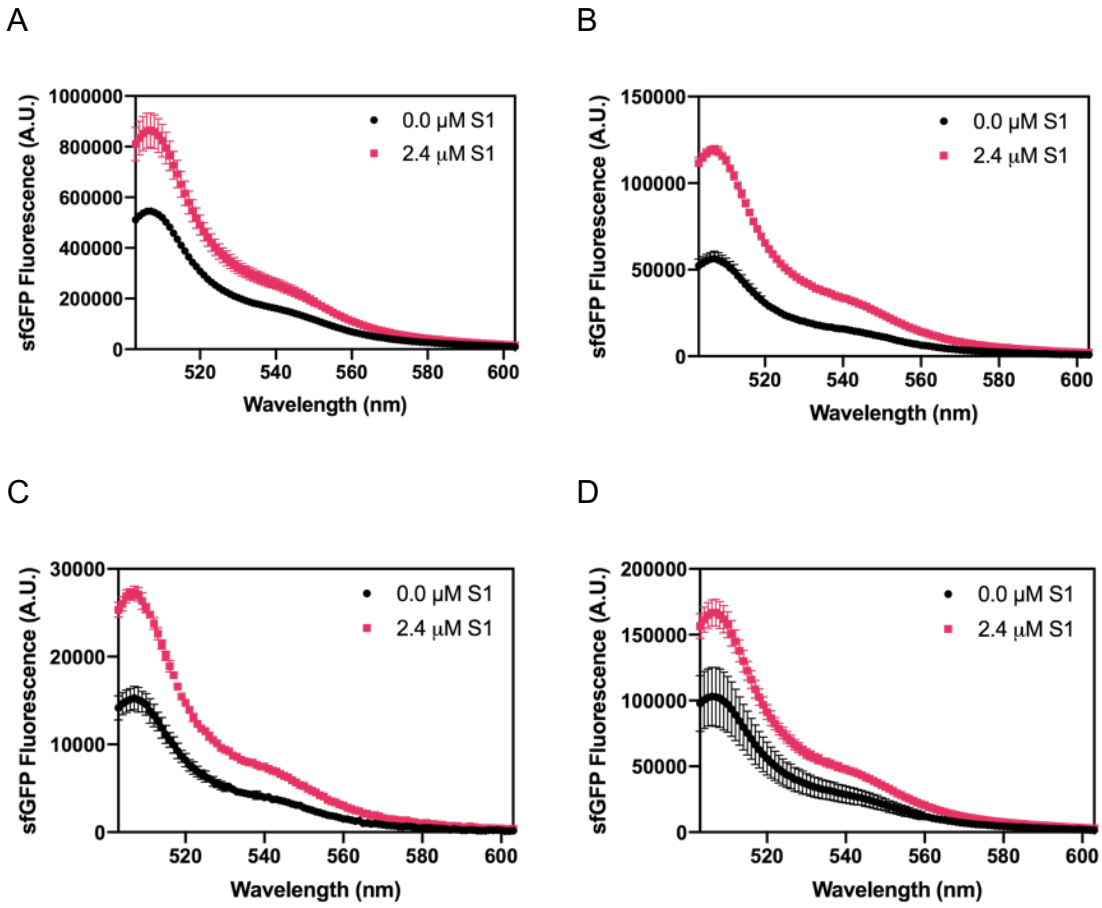

Figure A4-8: The addition of ribosomal protein S1 improves the in vitro reconstituted cell-free production of sfGFP in different 5'UTR contexts.

2.4 μM of ribosomal protein S1 was added to the TX-TL reaction in 1-fold excess relative to the ribosome concentration (2.4 μM). sfGFP was excited at 488 nm and emission scanned between 503-603 nm (λ_{max} = 508 nm). Negative control (without template) was subtracted from the emission spectra. (A) sfGFP expression is driven by the strong RBS BBa_B0034, (B) the medium RBS BBa_B0032, (C) the weak RBS BBa_B0033, or (D) by the highly structured PSIV IRES.