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Broadening the Application Range of Cell-Free Protein Expression Systems

Matthew Becker
mrbecke@clemson.edu

BROADENING THE APPLICATION RANGE OF CELL-FREE PROTEIN EXPRESSION SYSTEMS

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Matthew Becker
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Accepted by:
David Karl Karig, Ph.D., Committee Chair
Sarah Harcum, Ph.D.
Angela Alexander-Bryant, Ph.D.

ABSTRACT

Centralized protein manufacturing platforms make the delivery of needed therapies to places with limited infrastructure almost impossible. Cell-free protein expression systems, systems that utilize protein production machinery extracted from cells, offer these communities a viable protein expression platform that is robust and easily deliverable to the place of need. Much work in cell-free system engineering looks to increase the hardiness of cell-free system (CFS) components, like the extract and reaction buffer, needed to carry transcription and translation of gene therapeutic targets forward. Freeze- and air-drying of extract, reaction buffer, or both with certain additives, like sugar molecules, has been proposed with some success. However, when CFS components are lyophilized or air-dried together, the system's efficacy is significantly impacted when preserved over time and at non-ambient temperatures, which these systems will likely encounter in the field. More importantly, no methods have been proposed to improve cell-free system capacity outside standard reaction conditions. In other words, the operating range of cell-free systems is severely limited and needs to be addressed. Therefore, the purpose of this thesis work was two-fold. First, expand the operating range of cell-free systems and, second, better preserve CFS components.

To do so, a new experimental scheme was developed, namely the I3 scheme – identify, investigate, and integrate. First, climate-specific chaperones from extremophiles were *identified* from the literature in addition to gene regulatory elements controlling the chaperone's expression in the CFS. Second, these chaperones were *investigated* in CFSs to gauge whether they support CFS capacity over a wide operating range, specifically

high and low reaction temperatures. Third, these chaperones were *integrated* into the cell extract to elucidate their function in preserved and reacted CFSs.

These thesis results demonstrate that these CFS additives expand the operating range of CFSs relative to reaction temperature when expressed in parallel with the CFS or the cell extract. Additionally, it is demonstrated that these chaperones better preserve CFS components during air-drying and storage for up to 4 weeks. Together, these findings show that heterologous chaperones can function synergistically in CFSs. This work adds to the library of components at our disposal to make possible fieldable protein expression systems.

ABSTRACT

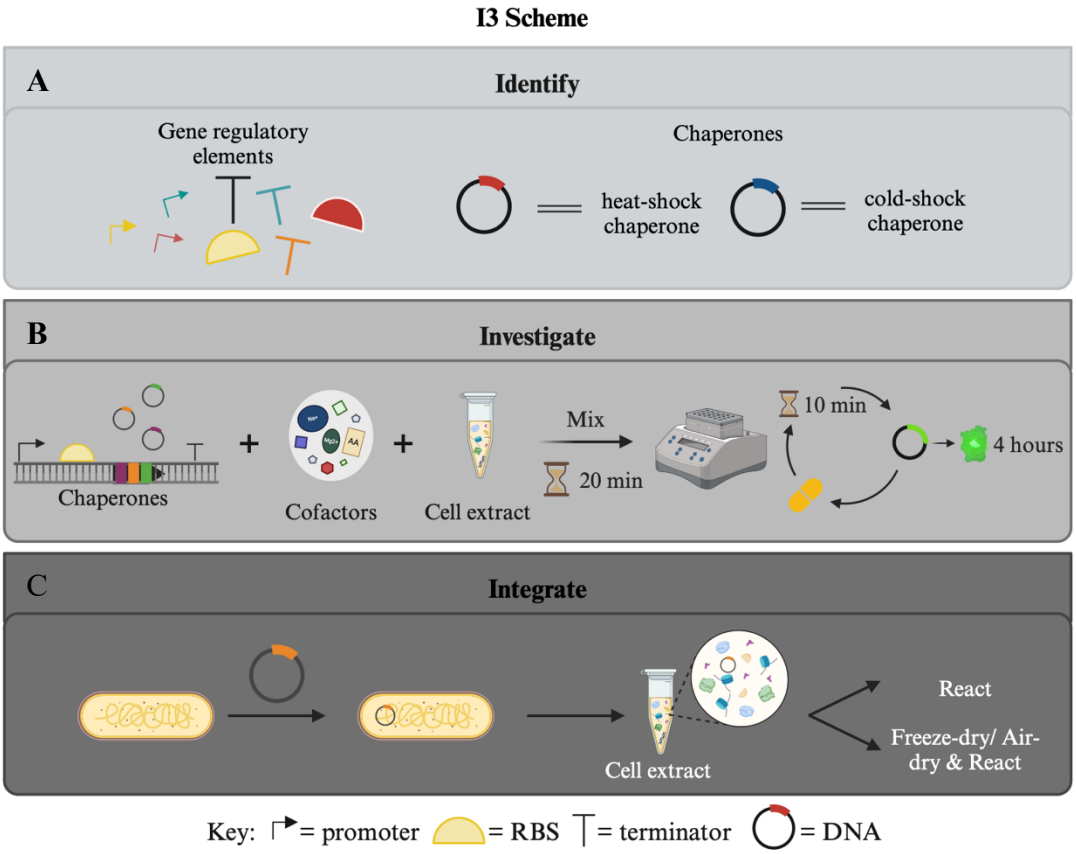

Figure 1: Graphical abstract. The I3 – identify, investigate, and integrate - experimental scheme is described. This scheme comprises three phases or steps. (A) First, gene regulatory components are strategically chosen to control the expression of the chaperone. (B) Second, chaperone utility is tested using a novel enhanced green fluorescent protein expression assay and (C) third, the chaperone components are recombinantly expressed in *E. coli* to generate extract for use in cell-free systems either lyophilized, air-dried, and/or reacted for improved reagent storage and reporter expression. Created with BioRender.com.

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CHAPTER ONE

INTRODUCTION

The emergence of cell-free gene expression (CFE) in the mid-20th century provided a pliable system and methodology to elucidate fundamental biological phenomena and certainties like DNA replication and the genetic code. More recently, CFE has been used for practical, commercial purposes, including, but not limited to, large-scale and point-of-use biomanufacturing. Additionally, CFE is used ubiquitously for the structural and functional study of proteins, colloquially referred to as proteomics, and, more broadly, applications relating to synthetic biology like biosensing. Simply put, CFE is a transformational platform that will inform and influence bioprocesses common to research laboratories and industries in the years ahead.

An emerging and notable arm of CFE, namely cell-free protein expression (CFPE), has undergone its technological renaissance, making producing functional protein products possible with a few simple steps. This technology can offset the limitations of conventional biomanufacturing platforms, specifically cell-based protein expression. Additionally, CFPE can significantly improve how we distribute functional protein products at home and overseas, aid resource-limited communities, or respond to emergent health situations like a pandemic.

Today, much, if not all, of the protein products used commercially are produced in-house at complex manufacturing facilities. These facilities must be maintained and operated by well-trained technical staff. Biopharmaceutical processes are not portable because of this fact, yet most end-products require cold-chain storage to and at the place

of sale [1,2]. This transport burden makes delivering needed therapies difficult to places and countries with limited infrastructure capacity and worsens our readiness to deal with global emergencies. Conversely, CFPE is remarkably versatile; comprised of just three independent components. These components are crude cellular extract, reaction buffer, and a DNA template encoding the desired protein. These systems can be assembled on-site, and their components, because of the scholarly work done by synthetic biologists, can withstand fluctuations in temperature, humidity, and so much more.

The work presented in this thesis lays out practical, straightforward methods to extend the versatility, practicability, and usability of CFPE systems, with a heightened focus on ensuring the protein expressive capacity of these systems at elevated temperatures. More specifically, this thesis identifies problem areas in cell-free technology, namely the operating range of cell-free systems and the combined preservation of CFS components. It also includes scientific means to solve these challenges, including novel methods and assays developed to gauge whether chaperone components, a novel cell-free additive, can aid CFS technology in this way. This experimental approach is far-reaching, with several CFPE system designs already imagined.

Thesis Overview

Despite the marked improvements in cell-free gene expression technology over the last decade, few researchers have focused on broadening the operating range of cell-free protein expression systems and, instead, focused on improving the preservability of cell-free system components like the extract and reaction buffer. Recent cell-free protein

expression (CFPE) papers, like those authored by Wilding and colleagues in 2019, look only at cell-free system preservation and not, for example, how environmental fluctuations like temperature might affect system expressive capacity once reacted. In other words, they carry the cell-free reaction forward at standard reaction temperatures after system preservation but not at temperatures these systems might experience in the field. This research gap is shared across other cell-free research publications tied to fieldable protein expression. Because of this fact, few CFPE methods are generalizable according to reaction environments and conditions. Therefore, the two aims of this thesis were to (1) expand the operating range of CFPE systems relative to temperature and (2) better preserve CFS components. These aims were achieved by incorporating chaperone components from extremophiles primarily related to cold and heat shock into cell-free systems. Additionally, a cell-free toolkit, or genetic library, comprised of these chaperone components and gene regulatory elements controlling their expression, like promoters, ribosomal binding sites, and terminators, was developed.

Protective gene components, namely chaperones, can be strategically chosen and successfully incorporated into cell-free protein expression systems for improved protein expression capacity and overall system utility.

The following thesis chapters outline two methods to incorporate chaperone components into cell-free protein expression systems. The first method describes a system-reaction setup allowing chaperones to be expressed in parallel with the target

protein gene. This method permits quick screening and study of chaperone components relative to their effects on protein expression and core function in hardening the CFPE system. The latter method outlines how to effectively develop crude cellular extract with the chaperone components to ease system setup and ensure all reaction resources are devoted to expressing the intended protein product, not the chaperone component. Together, these methods open the door to rapid screening of chaperone components and quick improvement of CFPE systems.

CHAPTER TWO

LITERATURE REVIEW

The utility of cell-free gene expression has significantly evolved since its advent in the late 1950s. Today, researchers are utilizing these highly dynamic, versatile, and adaptable systems to answer core scientific questions, advance functional protein production, and monitor ever-changing environmental challenges. In the following paragraphs, I highlight several emerging cell-free system applications, the components that make up cell-free protein expression systems, and the methods that like-minded researchers have developed to harden or ruggedize cell-free protein expression systems for expanded utility. Specifically, I look to methods that propose the addition of small molecule protectants or stabilizing agents, freeze- and air-drying methods, like lyophilization, and paper-based cell-free systems, to improve cell-free system expressive capacity in conditions of system stress. Additionally, I address the shortcomings of these proposed methods and offer an alternative approach, specifically the methods developed and articulated in this thesis, that make possible rapid improvement of cell-free system expressive capacity for any number of stress conditions.

2.1 Emerging Cell-Free System Applications

The utility of cell-free gene expression (CFE) is widespread, with applications in growing and fascinating scientific fields like biosensing, proteomics, functional protein and toxin production, functional genomics, and the prototyping of metabolic pathways. Here, I aim to summarize the work of a few notable synthetic biologists leveraging the uniqueness of CFE expression technology to transform scientific and commercial fields

and rectify knowledge gaps in our understanding of protein function, composition, and structure. While this list of applications is not exhaustive, I attempt to articulate the widespread utility of these systems and why these systems are so exciting to synthetic biologists.

2.1.1 Functional Protein Production

Cell-free protein expression systems (CFPES) are better suited to the expression and production of therapeutic protein when compared to traditional methods, primarily those that are cell-based. Users of CFPES have better control over biomolecule synthesis profiles since living cells are not in use. The reaction environment, then, is easier to tune according to the manufacturer's needs, like duration of reaction and total protein titers, to name a few. These systems can be decentralized and more easily shipped to their place of need, with little to no reliance on cold-chain transport [3,4]. Additionally, these systems can generate therapeutic targets that would otherwise be toxic to the cell. An excellent example of this is the anti-cancer drug Onconase [5]. Lastly, proteins can be generated with noncanonical amino acids or amino acids not belonging to the 20 standard amino acids, greatly expanding the number of drug treatments available to treat disease states [3,6].

2.1.2 Environmental Monitoring

Cell-free gene expression technology has provided an inexpensive means for individuals with limited infrastructure access or capital to test for environmental pollutants, like fluoride, that worsen health outcomes if ingested and, more broadly,

worsen the environment and its biodiversity. These environmental systems reliant on CFE technology have dramatically improved how we monitor containment molecules in the environment.

For example, riboswitches, a regulatory element responsible for either transcriptional or translational control that is part of the DNA template in cell-free systems, have been developed to successfully detect fluoride levels above 2ppm or 100 μM [7]. This system was shown to work better than commercially available test strips whose detection level is 10 to 100 ppm [7]. Moreover, this method costs significantly less than purchasing and operating traditional electrochemical devices for field testing of compromising analytes like fluoride, costing only forty cents per reaction [7]. Lastly, this CFE technology is more easily stored and shipped and less likely to lose its efficacy due to the employment of freeze-drying methods like lyophilization [7].

Together, CFE technology offers a unique, effective, and dynamic approach to tackling the many environmental challenges we face and is quickly becoming the preferred method of small molecule contaminant detection when compared to methods employing genetically engineered bacteria (GEB) or methods using expensive electrochemical equipment [7,8,9]. Additional environmental monitoring applications using CFE technology have been proposed and can be found in these journal articles [10].

2.1.3 Proteomics

Proteomics is integral in facilitating our understanding of protein structure and function [11,12,13,14]. In so doing, proteomics enables researchers to study diseases and identify therapeutic targets. It even enables clinicians, through clinical proteomics, to

diagnose diseases and track, in real-time, treatment responses [12,13,14]. Historically, researchers have relied on cell-based techniques to elucidate the function of protein components through transfection, selection, and cloning, all of which are *in vivo* expression techniques [11,12]. These processes significantly slowed research and hamstrung crucial efforts in documenting and cataloging proteins.

Today, cell-free gene expression (CFE) technology enables the study of proteins en masse, something not quickly done with cell-based technologies and methods. This technology has advantages over *in vivo* techniques since these systems lack host-cell constraints and permit parallel expression of proteins [11,12]. In addition, technologies complementing CFE technology in proteomics, like protein microarrays, can be paired with automation techniques, quickly expanding the number of proteins that can be studied simultaneously [13,15].

CFE technology has its challenges in the study of proteins. Historically, difficult-to-express proteins (DEPs), like multidomain proteins, could not be adequately folded in prokaryotic-derived cell-free systems like *E. coli*. In response to this challenge, researchers developed eukaryotic-derived cell-free systems, like the wheat germ system, to express DEPs effectively and efficiently [12,16]. Later protocols would support DEP expression in *E. coli*. For example, membrane-bound proteins can be made in *E. coli* extract if the said extract is supplemented with exogenous biomimicking membranes like liposomes and nanodiscs [17]. CFE technology will continue to have an oversized role in proteomics, as control of protein synthesis pathways and expression of said proteins is much easier in this system setup.

2.2 Extract

2.2.1 Source Strains

Extracts used in cell-free protein synthesis (CFPS) have been derived from prokaryotes and eukaryotes. For example, *E. coli* extract is used ubiquitously to synthesize soluble proteins. It has gained favor across laboratories and in commercial settings because CFPS systems (CFPSS) using *E. coli* extract are cost-efficient, well-established, able to produce high protein titers, and easily prepared and engineerable [17]. Historically, *E. coli* CFPSS were limited in the proteins and protein complexes they could make. For example, glycosylation of soluble proteins in *E. coli* CFPSS, prior to 2018, was not possible, so mammalian-based CFPSS, like Chinese hamster ovarian CFPSS, became available. *E. coli* lacked the necessary host machinery to glycosylate protein products. To overcome this challenge, researchers transformed *E. coli* with the necessary machinery to glycosylate protein products - glycan synthesis and oligosaccharyltransferase (OST) molecules [18,19]. This approach is much cheaper than eukaryotic CFPSS supplemented with microsomes containing glycosylation machinery and those CFPSS made from insect, human, or mammalian cells [19,20,21].

Still today, there are numerous advantages to using CFPSS made from eukaryotic extract. CHO cell extract, for example, is best suited for studying membrane-bound proteins since it contains the necessary machinery for improved membrane-protein solubility [17]. In CHO cell extract, supplementing liposomes and nanodiscs like in *E. coli* CFPSS is unnecessary [17,19]. Additionally, CFPSS originating from CHO cell extracts enable the quick study of DNA templates encoding for protein therapeutic targets

before CHO cells are transfected and cultured for large-scale biopharmaceutical production [17]. Together, these extracts and others like them have provided alternative, non-cell protein expression platforms that will continue to impact several industrial, commercial, and scientific practices.

2.2.2 Extract preparative methods

Cell culturing for extract preparation has remained essentially unchanged. Here, I speak to the evolving extract preparative methods following the initial cell harvesting step. Methods to prepare viable and robust crude cellular extract for CFPSS have significantly evolved since the early 1970s [22]. Expensive machinery, like the French press or impinge homogenizer, to lyse cells are no longer required. Instead, sonication can be used [23,24]. Such a change was deemed impractical by earlier published works, like those authored by Kigawa et al., albeit in 2004, due to believed heating and sample management constraints [25]. Since then, sonication for cell lysis has become more mainstream, with published work certifying its efficacy in generating robust extract [23,24].

Additional improvements to the standard protocol written in 1973 have been made to reduce the time and labor requirements to make the extract. Dialysis and later centrifugation steps meant to improve the storage buffer solution and reduce background expression for downstream experimental applications have all but disappeared, thanks, in part, to Kim et al. [22,26,27]. Kim and colleagues demonstrate that, after cell lysis, a low-speed centrifugation step, specifically 12,000 relative centrifugal force (RCF), and a 30-

minute incubation period at 37°C, significantly improve the translational activity of the lysate and significantly reduce the level of background expression by 34% [26].

Shrestha et al. improved Kim and colleagues' work by utilizing low-cost culture and lysis equipment, like shake flasks and ultrasonic homogenizers [24]. However, they stopped short of generalizing their findings to different source strains [23,24]. Kwon et al., in response, authored a notable publication elucidating best practices and optimization parameters for two *E. coli* strain types during the pre-and post-lysis steps, including the effects of cell density during harvest, resuspension volume, total sonication energy imparted to the sample, cooling time between sonication cycles, centrifugation speed following sonication, and run-off reaction time, the time needed for ribosomal disassembly from endogenous mRNA and the time needed for degradation of host DNA and RNA [23].

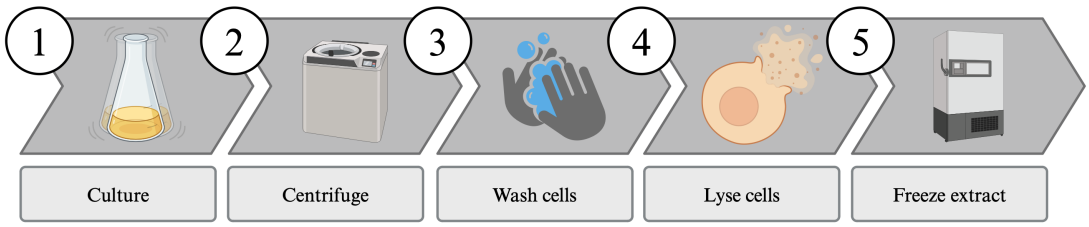


Figure 2: A simplified extract workflow. First, cells are cultured (1) until a sufficient cell density and growth rate is achieved. Next, cells are harvested (2), washed (3), and lysed (4) to obtain highly productive cell extract, then frozen (5). Created with BioRender.com.

2.3 Reaction Buffer

Reaction buffer comprises several cofactors, like energy and salt molecules, that carry transcription and translation forward in coupled transcription-translation cell-free systems. Traditionally, reaction buffer was made up of these components: salts, energy

molecules, buffer, amino acids, crowding and reducing agents, and tRNA [28]. Now, advanced systems, like the cytomim system introduced by Jewett and colleagues, mimic intracellular environments by incorporating native *E. coli* machinery and small molecules that permit equivalent system efficiencies and protein titers to traditional reaction buffer schemes but at a significantly reduced cost [28,29].

Early cell-free batch reactions relied on high-energy phosphate molecules to push translation forward. While this system achieves sufficient protein output, it is severely hampered by the build-up of inorganic phosphates that dampen magnesium concentration and contribute to a worsening pH reaction environment. Notably, Kim and colleagues have demonstrated that inorganic phosphate concentrations exceeding 30 mM contribute to CFE reaction malaise [30]. These inorganic phosphate concerns can be solved by employing the PURE system, which lacks endogenous phosphatases found in extract known to degrade high-energy phosphate molecules nonproductively, or by using continuous-exchange processes to rid the reaction environment of inorganic phosphates [30,31].

Today, substrate-level phosphorylation is not the only way to power CFE reactions. ATP-regenerating systems, like the PANOx and the cytomim system, have been produced. These systems utilize host metabolic processes by supplementing CFE reactions with specific cofactors and small molecules, like nicotinamide adenine dinucleotide (NAD) and coenzyme A (CoA), meant to power the conversion of pyruvate to acetyl phosphate as seen below [29,30,32]. These system changes offered by Kim and Swartz sought to reduce the number of exogenous enzymes and secondary energy sources

needed to carry translation forward in CFSs. In other words, they sought to utilize already present energy pathways native to cells to carry forward the energy-intensive process known as translation. The cytomim system, as described above, is simply an improved version of the PANOx, with the same motivation – mimicking the cell by using intracellular ATP-regenerating pathways [29]. The cytomim differs in these ways. 'Unnatural' components, like HEPES-KOH, a buffer routinely used in CFS workflows, were either removed entirely or supplemented with natural ones [29]. In this instance, the buffer was no longer needed as the pH of the CFE reaction remained relatively unchanged [29]. Additionally, crowding agents, like PEG8000, meant to improve molecular interactions, were replaced with polyamines spermidine and putrescine [29]. Lastly, pyruvate was used as the energy source, not PEP [29].

While these systems are notable and remarkably innovative, the reaction buffer scheme used in these thesis CFE reactions closely resembles that of the PANOx system with minor changes. For example, creatine phosphate, not phosphoenolpyruvate, is the secondary energy source, and folinic acid is added for formyl methionine synthesis, an amino acid used in prokaryotic translation. Additionally, the reaction buffer scheme used for this thesis lacks NAD and sodium oxalate, the latter meant to limit PEP synthetase [33]. Lastly, magnesium acetate concentrations were optimized according to each extract batch used for CFE reactions, as it has been shown that magnesium concentration has appreciable effects on the translation activity of cell-free systems [34].

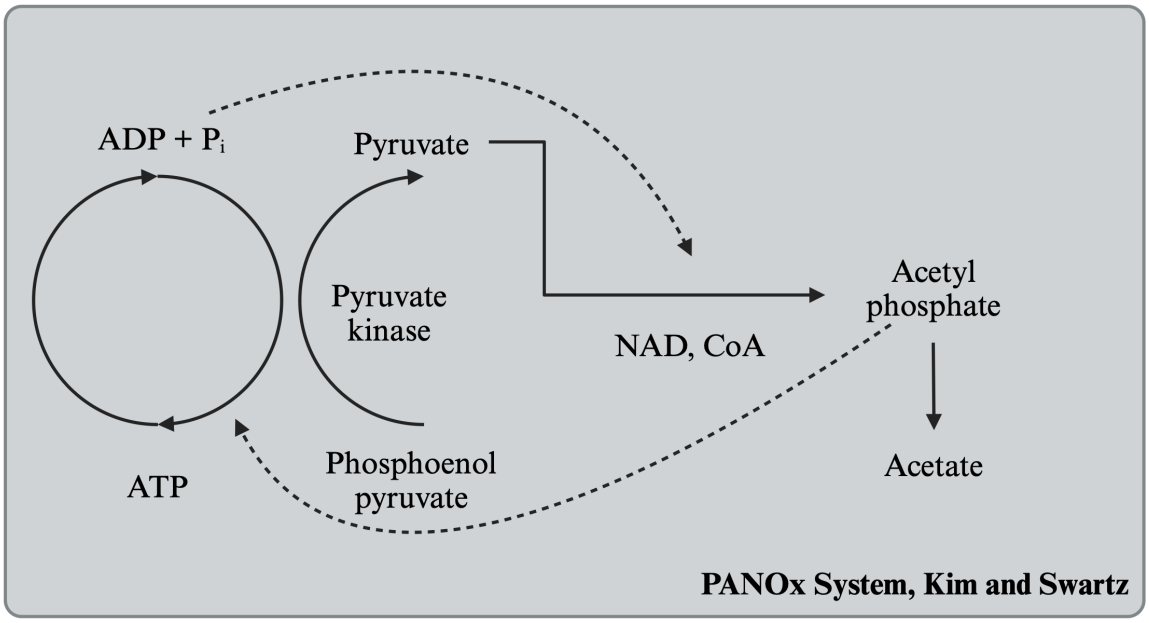


Figure 3: The PANOx system developed by Kim and Swartz is shown with minor detail [30]. Created with BioRender.com.

2.4 Methods to ruggedize cell-free systems

2.4.1 Lyophilization

Traditionally, reaction mixture components for CFPS reactions, like the extract and reaction buffer, are stored at -80°C, the standard storage temperature, to maintain functionality. This storage requirement severely limits the applicability of these systems and, consequently, the capacity to remedy the challenges of cell-based protein expression; the principal among them is cold-chain storage. As a result, lyophilization of either the cell extract or reaction buffer was proposed to improve CFPS system preservation at non-standard storage temperatures and to increase the amount of material that can be stored at one time - also known as the density of stored product [35,36,37,38].

Smith and colleagues, for example, demonstrate that lyophilization can have appreciable effects on extract efficacy when stored at non-standard storage temperatures, such as 27°C [36]. More specifically, they show that lyophilized extract, when stored at 27°C for 90 days, maintains 20% of its original effectiveness compared to the non-lyophilized extract, or extract in the aqueous phase, which loses almost all protein expression capacity following 30 days at room temperature [36]. To elucidate why this is the case, the authors look to bacterial contamination. They report significant bacterial contamination in the aqueous phase extract, enough to affect system protein output [36]. Interestingly, Smith and colleagues report similar lyophilized extract efficacy even when lyoprotectants, like sucrose, are added. They surmise that the sucrose-deficient extract contains endogenous machinery that aids in protein stabilization during drying, most likely a native chaperone or other small molecule [36]. This finding maps with other published works as recently as 2019 [37]. Lyoprotectants have been shown to not do that much during the lyophilization process. Their effects are felt when the extract is preserved over extended periods [4,36,37].

Smith et al. further evaluated the stability of reaction buffer (RB) components, given the purported instability of high-energy phosphate molecules, like phosphoenolpyruvate (PEP) and nucleoside triphosphates [36]. They report different GFP production profiles for those CFE reactions using lyophilized RB components and CFE reactions using traditional, aqueous phase RB components at room temperature, with the lyophilized RB components achieving higher relative GFP yield at 30 days [36].

These findings by Smith and colleagues bolster this technology's appeal in delivering therapeutic targets to places with capital limitations for the transport or distribution of therapies. However, it falls short of addressing a core need identified by industry and partners, a one-pot CFE system. A so-called one-pot CFE system that contains both the extract and RB components is ideal, as it cuts down on the number of moving parts and makes system assembly easier. Smith and colleagues fail to show that such a system is possible by demonstrating a significant drop in CFE performance when extract and RB are lyophilized together [36]. Additionally, they failed to test this combined lyophilized system at temperatures 4 and 27°C, citing their finding of extract degradation at elevated temperatures [36]. These results suggest a new approach for CFE ruggedization is needed and, thus, the motivation for our chaperone-inspired cell-free system.

2.4.2 Lyoprotectants and endonuclease inhibitors

Prior research has shown that specific small molecules and polymers, like trehalose and polyvinylsulfonic acid (PVSA), can positively affect CFE performance [4,38]. Small molecules, like trehalose, a disaccharide, often serve as lyoprotectants during the lyophilization of the extract, reaction buffer, or both – meant to improve the sample's viability during the freeze-drying process. Interestingly, lyoprotectants, like sugars, have been shown to significantly affect the stability of lyophilized CFPS components, specifically proteins, at elevated temperatures over time and even during air-drying processes [4,37]. It has been hypothesized that sugar molecules function to stabilize protein components in lyophilized CFPSS by one of two processes – water

replacement and vitrification [37,39,40]. Small sugar molecules in the first hypothesized instance are thought to hydrogen bond with protein components during freeze-drying, thereby stabilizing protein components [37,39,40].

Additionally, because of their small, flexible structures, disaccharides can interact with protein components better than long-chain, polymeric sugars like dextran, making them better candidates for cell-free system preservation [37,39,40]. However, in vitrification, sugar glasses with high glass transition temperatures, or T_g's, antiplasticized with antiplasticizing agents, those molecules with a higher T_g, significantly reduce the molecular mobility of the protein or, in this case, cell-free system components, improving protein and system stability during preservation [37,39,40]. The results of Wilding and colleagues best articulate this finding. For example, their sugar-glass matrix comprised of ficoll, maltitol, the linker, and DMSO, later referred to as FMD, when added to the extract, lyophilized, and reconstituted, performed better than trehalose-preserved cell-free reactions at all four experimental temperature conditions ranging from 4 to 50°C and all four-time points [37]. This result no longer becomes true when lyoprotectants like FMD are added to a reaction mixture (extract + reaction buffer) and lyophilized. Such a system significantly underperforms a system only containing dextran at nearly all temperatures tested except for 4°C [37]. While interesting, the authors in this piece fail to test trehalose in CFSs where the extract and reaction buffer are lyophilized together, citing their initial findings in which the trehalose system significantly underperforms that of the others. They acknowledge, however, that trehalose-inspired CFSs maintain their performance no matter the degree or time point tested, albeit significantly lower than the other systems. A

notable trehalose-inspired CFS is the one authored by Karig and colleagues. They put forward an air-drying method that limits our reliance on relatively new – new to the cell-free landscape - and costly preservation methods like lyophilization and achieves sufficient protein output [4].

Aside from the extract and reaction buffer, the number of mRNAs available for translation in solution is integral to CFS success. To that end, Earl and colleagues propose adding PVSA to CFSs to stabilize mRNA molecules by quenching endogenous extract nucleases, specifically RNases [38]. They show that PVSA in a decoupled transcription and translation system (TTS) enables higher reporter expression [38]. However, in coupled TTSs, the one of interest, these authors show that PVSA inhibits CFE performance and subsequent reporter yield [38]. Practically, then, this effort would not be helpful for fieldable protein platforms, and thus, the need for an alternative solution.

These authors put forward methods that allow for improved extract efficacy during and after lyophilization at elevated temperatures but fall short in identifying a CFS additive that, when lyophilized with extract and reaction buffer components, allows for significant CFE performance at elevated temperatures [4,36,37,38]. Wilding and colleagues acknowledge this but say that system preservation at 50°C for most applications is unnecessary [37]. Chaperone addition to CFSs, we believe, can address both needs – enable improved stability of the reaction mixture and transcribed mRNA molecules.

2.4.3 Paper-based cell-free gene expression

Paper-based CFS platforms are used in sensing and diagnostic applications outside protein production. For example, Pardee and colleagues imagined a paper-based CFS that can produce a colorimetric output, specifically the LacZ reporter, visible to the naked eye by employing toehold-based mRNA sensors to sense for two strains of Ebola that differ by only three nucleotides [41]. This work is significant as it proposes a method for easy system development, relies only on sequence information, and offers quick deployment of sensing operations in response to an emerging pathogen [41]. However, no consideration was given to where these sensors might operate. Most results rely on carrying reactions forward at standard reaction conditions like incubation at 37°C for system expression, or the systems are reconstituted 24 hours following freeze-drying. No explanation was given for how these systems might perform if preserved over time or used in substandard environments. This is where chaperone addition to such a system might prove beneficial and a goal of this thesis work.

Lin and colleagues propose something like that of Pardee's seminal work. They developed a paper-based CFS that can successfully sense the environmental pollutant arsenic and sense the Pseudomonas autoinducer, -3-oxododecanoyl-homoserine lactone (3OC12HSL), with two colorimetric outputs resulting from the enzymes, Lac X and XylE [42]. Interestingly, all but one lyoprotectant, sucrose, improved the paper-based CFS sensing ability following freeze-drying, indicating that lyoprotectants, like Trehalose, can prohibitively impact CFS performance, even during sensing operations [42]. These findings again suggest that alternative CFS protectants are needed during lyophilization

and beyond for applications outside therapeutic protein production, such as sensing compromising molecules.

Lastly, Hunt and colleagues developed a notable point-of-care COVID-19 biosensor. They demonstrate that their freeze-dried paper-based CFS with murine RNase inhibitor (mRI) can reliably detect mRNA target molecules at a concentration of 40 nM [43]. Additionally, they hypothesized that their low-density polyethylene cassette housing the paper-based CFS will enable improved system preservation and easier distribution [43]. They fail to demonstrate that the former claim is valid and offer only that this system performs well at ambient temperatures. A CFS additive like a chaperone might better serve systems like this one, primarily when functioning at non-ambient temperatures.

CHAPTER THREE

CHAPERONE SELECTION, PLASMID DESIGN & LIMITATIONS OF THE *E. COLI*-DERIVED CELL-FREE SYSTEM

3.1 Introduction and statement of purpose

Chaperone proteins have long been implicated in affording specific environmental stress tolerances to species living in environments once thought inhabitable. For example, *Deinococcus radiodurans* can withstand ionizing radiation several degrees higher than their prokaryotic counterparts [44]. Historically, chaperone proteins have been used recombinantly to improve organismal capacities. For example, cold-shock proteins from *Escherichia coli* and *Bacillus subtilis*, namely CspA and CspB, have improved grain yields where water is scarce [45]. Examples like this one are plentiful and cover many biotechnological arenas outside agriculture, even cell-free [46]. These notable and continued findings of chaperone protection led to the question: Can chaperone proteins be used to improve the operating range of CFPE systems and aid in preserving CFS components?

The objectives of this chapter, then, were three-fold. First, chaperone candidates were identified that would improve the operating range of *E.coli*-derived cell-free systems relative to temperature. Therefore, the search was limited to chaperone components previously demonstrated to confer temperature stress protection to *E. coli*. Second, chaperone plasmids were developed to support their robust expression in *in vitro* experiments, as seen throughout this thesis. This was achieved by strategically selecting and incorporating gene regulatory elements into the plasmids, including the promoter,

ribosomal binding site, and terminator. Third, baseline characterization of these systems was performed to elucidate system fail points outside cell-free reaction temperature norms.

3.2 Chaperone Selection and Plasmid Design

3.2.1 Chaperone Selection

Chaperone components were strategically chosen to afford the CFS specific stress-related tolerances, like cold and heat tolerance. All but two chaperones, DHN1α and CAHS 94063, were shown to promote *E. coli* viability in temperature stress conditions upon *E. coli* transformation with these chaperones. For example, Yang and colleagues demonstrate that *E. coli*, transformed with the chaperone component, WZY2, a dehydrin, significantly improved *E. coli* viability at 0°C and 50°C [47]. Na and colleagues demonstrate something similar for PtDRG1, a desiccation response gene from *Pyropia tenera*, an alga [48]. At 46°C, transformed *E. coli* were shown to have a higher growth rate than the control *E. coli*, which lacked the chaperone plasmid [48]. Lastly, the chaperone CsLEA11, a dehydrin native to the cucumber, *Cucumis sativus*, when recombinantly expressed in *E. coli*, was shown to confer heat and cold tolerance and improve cell viability [49]. These findings suggest that chaperone activity in *E. coli* supports cell growth and cell survival at extreme temperatures and might allow for improved robustness of a CFS derived from transformed *E. coli* containing the heterologous chaperone. That is to say that chaperones might function downstream of extract preparation and aid in CFS success. For example, these chaperones might aid in

preservation efforts or the expression of targeted proteins at elevated temperatures. Such effects were examined in Chapters 4 and 5 of this thesis.

Another candidate is DHN1α, a dehydrin native to *Vitis vinifera*, a grape. Rosales and colleagues show that this chaperone can support specific proteins and enzymes like lactate dehydrogenase (LDH) at low temperatures [50]. Additionally, they showed that this chaperone protects malate dehydrogenase (MDH) from dehydration and even has antifungal properties against a fungus known to affect grapes, *Botrytis cinerea* [50]. This chaperone was selected for its abiotic and biotic stress protection functionalities. Interestingly, Ferrer and colleagues demonstrate something similar, although the chaperone they study is not native to *E. coli*. They show that the cold-shock chaperone, Cpn 60, and its co-chaperone, Cpn 10, native to *Oleispira antarctica*, improved *E. coli* cell growth at low temperatures, primarily by aiding protein folding [51]. They hypothesized that these chaperones might function to improve *E. coli* cell growth by some other pathway not yet elucidated [51]. Like Ferrer et al.'s work, this thesis investigated whether chaperone functionalities can be extended to non-native systems, like CFSs, under non-traditional environmental and reaction conditions.

Lastly, it has been demonstrated that the protective protein CAHS 94063 desiccation function depends on interactions with solutes like trehalose [52]. This finding is useful since lyoprotectants like trehalose have been used in prior studies to bolster CFS functionality during the freeze-drying process and later preservation efforts. Together, these chaperones might offer protections yet to be afforded to CFSs and, in so doing, enable fieldable protein expression systems.

Host Species	Protein Name	Protein type	Accession #	Reference Article
Pyropia tenera	PtDRG1	Desiccation response gene (DRG)	KX610933	Na et al.
Vitis vinifera	DHN1α	Dehydrin (DHN)	JN689936	Rosales et al.
Triticum aestivum	WZY2	DHN	EU395844.2	Yang et al.
Cucumis sativus	CsLEA11	DHN	XM_004150027.3	Zhou et al.
Hypsidbius dujardini	CAHS 94063	DRG	P0CU50	Nguyen et al.
Oleispira antarctica	Cpn 60/10	Chaperonin (Cpn)	Q8KM31.1/30.1	Ferrer et al.

Table 3.21: Overview of chaperones used in this thesis.

3.2.2 Plasmid Design

Plasmids expressing the chaperone and reporter components used in these experiments were designed and constructed using the software services IDT DNA, De Novo DNA tools (Salis), and SnapGene. IDT DNA was used for codon optimization of the targeted chaperone sequences so that they could be expressed efficiently in *E. coli*. Robust ribosomal binding sites were designed *in silico* with De Novo DNA tools native to SalisLab. Lastly, SnapGene was used to piece each plasmid part together.

Each plasmid used in these experiments can express its coding sequences, or CDS, from a constitutive T7 promoter, a promoter native to T7 bacteriophages [53]. Such a setup allows for strong expression of each chaperone and reporter component in *E. coli* strains that contain the lambda DE3 phage construct encoding for T7 RNA polymerase [53]. Additionally, the same is true of cell-free systems whose lysate is derived from this type of *E. coli* strain, the lysate type used throughout this thesis, specifically BL21 Star

(DE3) (Invitrogen, Waltham, MA) lysate. Next, ribosomal binding sites were designed *in silico* with SalisLab to enable high transcript translation efficiency, and the terminator, BBa_B1006, found in iGEM's Registry of Standard Biological Parts, was chosen for its high termination efficiency at 99.4%, as measured by Cambray et al. [54]. Lastly, an operator sequence, TetO, the TetR-binding domain, was added downstream of the T7 promoter so that expression of the chaperone components could be repressed if, in solution, they were prohibitive to CFE performance.

Regulatory element	**Type**	**Sequence**	**Reference Article**
Promoter	T7	TAATACGACTCACTATAGG	Studier et al.
Operator	TetO	TCCCTATCAGTGATAGAGA	Karig et al.
Terminator	BBa_B1006	AAAAAAAAACCCCGCCCCTGACAGGGCGGGGTTTTTTTT	Cambray et al.

Table 3.22: Summary of shared regulatory elements across all six chaperone components.

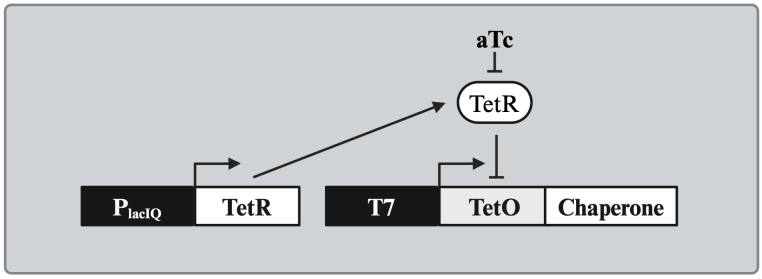


Figure 3.21: P_{lacIQ} and T7 Tet system. TetR is expressed from the constitutive P_{lacIQ} promoter and represses the operator TetO and chaperone expression. The repressor TetR can be inhibited by adding anhydrotetracycline (aTc). Created with BioRender.com.

3.3 *Escherichia Coli*-derived cell-free system limitations

3.3.1 Methods and Materials

3.3.1.1 Extract

Three extract batches were used to generate the results below. A detailed protocol explaining how the BL21 extract was prepared can be found in A-2. The E60 and E80 extract batches were prepared according to the methods proposed by Karig et al. [4], with changes to how the cells were sonicated. The '60' and '80' denotations correspond to the amplitude setting used during sonication. Panels A, B, and C were generated using the extracts E60, E80, and BL21, respectively.

3.3.1.2 Reaction Buffer

The reaction buffer methods described in A-1 were used here, with minor changes to the magnesium acetate concentrations specific to the type of system used. For the E60 and E80 CFSs, a magnesium acetate concentration of 32 mM was used. For the BL21 CFS, a magnesium acetate concentration of 28 mM was used.

3.3.1.3 Enhanced green fluorescent protein (EGFP) expression assay and normalization scheme

The EGFP expression assay and normalization scheme indicated in A-3 was used here.

3.3.2 Results

Three conventional cell-free systems (CCFS), including 'E60', 'E80', and 'BL21', were evaluated across a wide temperature range to identify which temperatures

cause these systems to fail and where chaperones might be helpful. The ‘E60’ CCFS, shown in panel A, Figure 3.3.21, maintains only 21% of its expressive capacity at 40.2°C and only 5% at 41.4°C. The ‘E80’ CCFS shown in panel B performs better than the ‘E60’ CCFS, with 40% of its expressive capacity maintained at 40.2°C and 28% at 41.4°C. Only at 42.1°C does the ‘E80’ CCFS fall below 5% of its optimal system performance at 37°C. The ‘BL21’ CCFS, seen in panel C, follows similar reporter output as a function of temperature trends. For example, at 41.1°C, only 16% of the system’s expressive capacity is maintained, with only 4% observed at 42.7°C. These findings continue past 42°C for the ‘BL21’ CCFS, as only 1% of system expressive capacity was maintained at 45.9°C.

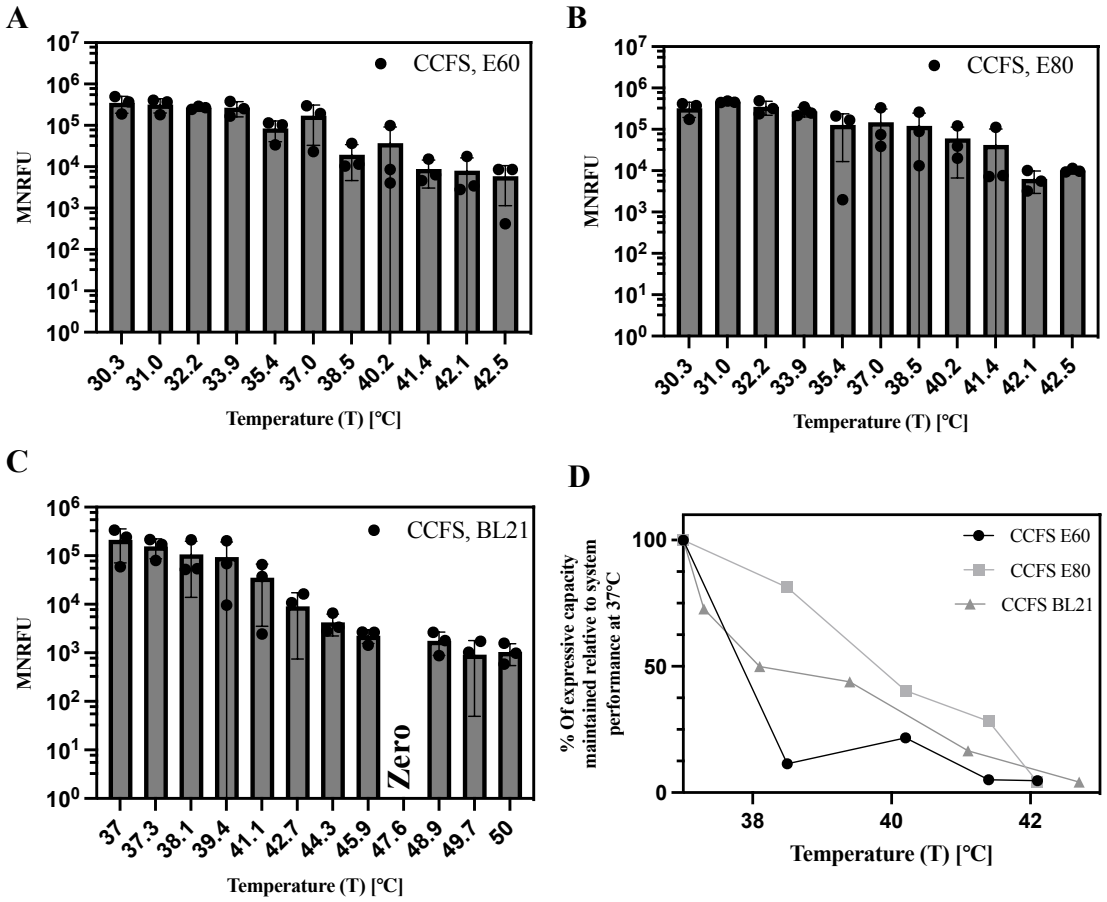


Figure 3.3.21: Conventional cell-free system analysis at standard and non-standard reaction temperatures across three extract batches prepared uniquely and separately. (A) Reporter expression at different temperatures using the 'E60' extract. (B) Reporter expression at different temperatures using the 'E80' extract. (C) Reporter expression at different temperatures using the 'BL21' extract. (D) Percent of expressive capacity maintained at elevated temperatures relative to system performance at 37°C. Reported values represent mean fluorescence from triplicate samples (given by the number of dots), and error bars represent standard deviation.

3.4 Discussion

Chaperones were strategically chosen from the literature based on their stress-affording capabilities to their native or non-native host. The focus of stress protection was temperature-related protection. It was thought that these same chaperones might afford similar protection to CFSs derived from *E. coli*. The chaperone coding sequences were used to develop plasmid maps that could be recombinantly introduced to *E. coli* and afford constitutive expression of the chaperone components. Additionally, orthogonal control of the chaperone can be achieved by expressing the TetR protein should chaperone concentration prove prohibitive in reaching higher cell-free system capacities.

It was found that CCFSs, regardless of how the extract was prepared and at which sonication amplitude – the degree of longitudinal movement of the sonicator probe in a sample - lose nearly all their expressive capacity at temperatures exceeding 42°C, with only 4% maintained. Thus, this downward trend of CFS expressive capacity is due to temperature effects, not system, specifically extract, preparation. These baseline characterizations guided later methods and assay development in this thesis. For example, we knew at which temperatures chaperones were needed. In summary, this chapter outlined how chaperone components can be chosen and incorporated into viable plasmid backbones for expression in CFSs. It also delineated where chaperones are needed to improve a CFS's operating range and subsequent reporter expression at those once-thought-prohibitive reaction temperatures or environments.

CHAPTER FOUR

EVALUATION OF CHAPERONE COMPONENTS IN CFSs UNDER TEMPERATURE STRESS

4.1 Introduction and statement of purpose

Cell-free system technology has come a long way in the past ten years. Cell-free researchers have accomplished notable feats in better preserving cell-free system components like the extract and reaction buffer [4,37]. However, no one has yet to tackle the more pressing and practical challenge inherent to these systems operating in the field, a target application of this technology – their operating range. Countless examples exist of cell-free systems being preserved at elevated temperatures for long periods. Yet, when these systems are reconstituted, they are reacted at standard reaction conditions, i.e., those conducive to the system and the target protein, usually 30°C or 37°C [37]. These traditional reaction environments are unlikely to be encountered in the field, and why this research is so pressing.

Two goals for this chapter were proposed to overcome this research gap in cell-free technology. First, we verified whether chaperones could expand or improve the operating range of CFSs relative to temperature and identified which chaperone plasmid DNA (CPD) concentrations were most conducive for this task. In other words, this task elucidated which CPD concentrations were beneficial to cell-free environments and which concentrations were prohibitive – if there were such a range. Second, an EGFP expression assay was developed to aid current and future chaperone studies looking to

test other chaperones and their functionalities relative to a wide range of reaction temperatures.

4.2 Methods and Materials

4.2.1 Transformation & DNA purification/extraction

The transformation protocol and DNA purification/extract methods indicated in A-4 were used here.

4.2.2 Extract and reaction buffer

The extract to generate these data came from two extract batches, E60 and BL21. The step-by-step process to create the extract batch, BL21, can be found in A-2. The E60 extract was used to generate all results in Figures 4.31 and 4.32, and the BL21 extract was used to generate all results in Figure 4.33. The reaction buffer scheme noted in A-1 was used here.

4.2.4 EGFP expression assay and normalization scheme

Two EGFP expression assays were used. The first EGFP expression assay described in Figure 3.31, panel A, was used in initial CFE experiments to screen chaperone components. Initially, 10, 15 and 45 μL CFE reactions were used, each comprising roughly 77% reaction mixture and 23% DNA. Chaperone DNA was added to the reaction mixture at the specified concentrations below and was incubated for 30 minutes at room temperature. Samples were placed in the thermocycler, and reporter DNA was added. The samples were reacted for 12 hours, after which kanamycin (100

μg/mL) was added to arrest reporter expression. Fluorescence was assayed in a Biotek Cytation 5 as described in A-3.

For the second assay, referred to as the novel EGFP expression assay in later thesis sections, 15 μL CFE reactions were used. Panel D in Figure 4.32 and all panels in Figure 4.33 were generated using this assay. Additionally, while the chaperone components were reacted with the reaction mixture for 30 minutes, the last 10 minutes of this reaction was done in the thermocycler at the desired experimental temperatures, not room temperature. The fluorescence scheme indicated in A-3 was used here.

4.3 Results

To gauge chaperone effects in CFSs, all six chaperones were expressed in these systems at low or high temperatures, and fluorescence output was assayed. All but two chaperones effectively broadened these systems' operating range in low- and high-temperature directions. The effective chaperones include Cpn 60/10, WZY2, CsLEA11, and PtDRG1. For example, the cold-shock chaperone CFS, Cpn 60/10, performs better at a working plasmid DNA concentration of 10 ng/μL than the EGFP and mCherry controls at 10°C. Compared to the EGFP control, which has no chaperone component expressed, Cpn 60/10 improves reporter expression by more than 13 times, and compared to the mCherry control, only 1.06 times (Figure 4.31, panel B). Initially, it was hypothesized that the mCherry control would perform considerably worse than the EGFP control since energy resources would now be directed to transcribing and translating the mCherry protein in place of EGFP. Instead, the mCherry control achieved higher reporter expression than the EGFP control in almost every result, irrespective of temperature

(Figures 4.31 and 4.33). It was thought that the mCherry control's heightened system performance was due to mCherry fluorescence spillover in the green channel, giving rise to reported higher EGFP fluorescence and perceived system benefit. Upon further investigation, we found that this was not the case. Much like oxygen-detoxifying mechanisms related to bioluminescence, it could be that the mCherry protein, upon excitation, undergoes collisional quenching with oxygen, improving the reaction environment via oxygen-detoxification. This may explain why the mCherry control performs much better than the EGFP control.

In panels C and D in Figure 3.31, the chaperone components, CsLEA11, WZY2, and PtDRG1, at a working chaperone plasmid DNA concentration of 5 ng/μL, 3.33 ng/μL, and 10 ng/μL, respectively, are shown to improve CFS performance by ~3.4x, ~1.7x, and ~2.3x, respectively, when compared to the negative EGFP, CCFS control. Significance, though, was reported for only two of three chaperone components, specifically CsLEA11 and WZY2. Lastly, it is shown that certain chaperone plasmid DNA concentrations, especially those at comparatively low and high levels, specifically 2.5 ng/μL and 20 ng/μL, negatively impact CFS performance (Figure 3.31, panels C and D). This finding is shared in subsequent thesis chapters, elucidating why Tet repressible promoters are needed when generating chaperone-transformed extract.

The generic EGFP expression assay shown in Figure 4.31, panel A, was not able to adequately capture temperature effects on CFS performance due to sample heating constraints. In other words, the samples were not given adequate time to reach the desired experimental temperature. This result was borne out by adjusting CFE reaction volume

and temperature, as seen in Figure 3.32. By accounting for this assay discrepancy, WZY2, at a working concentration of 7.5 ng/μL, improves EGFP expression by more than 5 times compared to the EGFP, CCFS control (Figure 3.32, panel D). Later experiments using the novel EGFP expression assay, as illustrated in Figure 3.33, in panels A and B, more accurately demonstrate the effects of chaperones in CFSs. Additionally, since earlier range-finding efforts meant to elucidate the best chaperone plasmid DNA concentrations proved helpful, the CsLEA11 CFS outperforms the EGFP control by a thousand-fold at 39.8°C (Figure 4.33, panel A). Though less dramatic of a change, the PtDRG1 CFS now reliably performs better than the EGFP control at high temperatures. Specifically, the PtDRG1 CFS outperforms the EGFP control by ~ 9.0 and ~5.6 times at 39.8°C and 42°C, respectively (Figure 4.33, panel B).

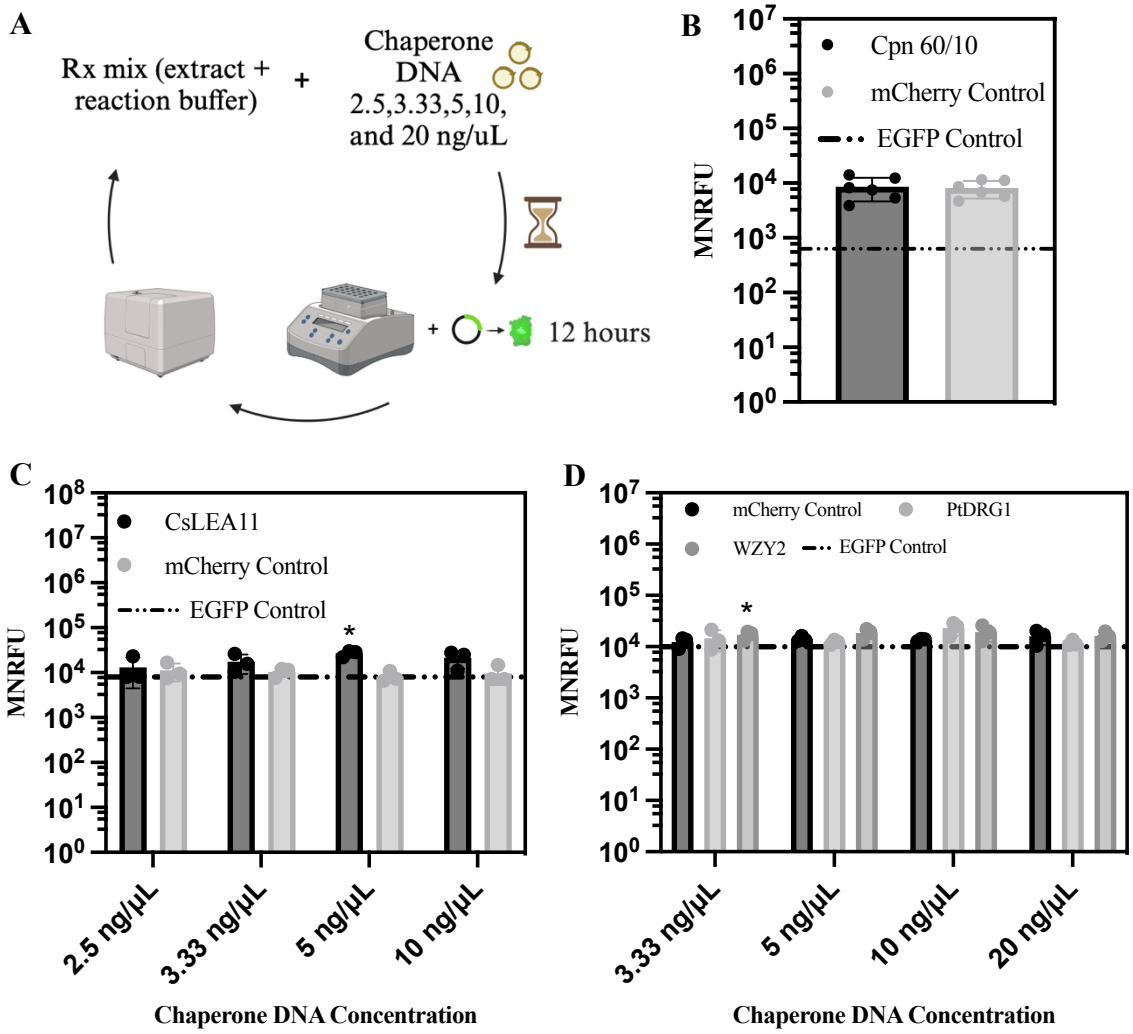


Figure 4.31: The effect of chaperone expression in CFSs at low and high temperatures for a 'generic' EGFP expression assay. (A) Schematic detailing the steps taken to generate this panel of results. (B) Cpn 60/10, a cold-shock chaperone, was evaluated in 10 µL CFE reactions at 10°C. (C & D) CsLEA11 and WZY2, both dehydrins, were evaluated in 10 µL and 15 µL CFE reactions, respectively, at 45°C with varying DNA concentrations. Reported values represent mean normalized fluorescence from 3 (C and D) or 6 replicates (B) (given by the number of dots), and error bars represent standard deviation. Reported statistical significance was found using a standard, unpaired Student's t-test, correcting for unequal variances. '*' indicates a p-value less than 0.05. Panel A was created with Biorender.com.

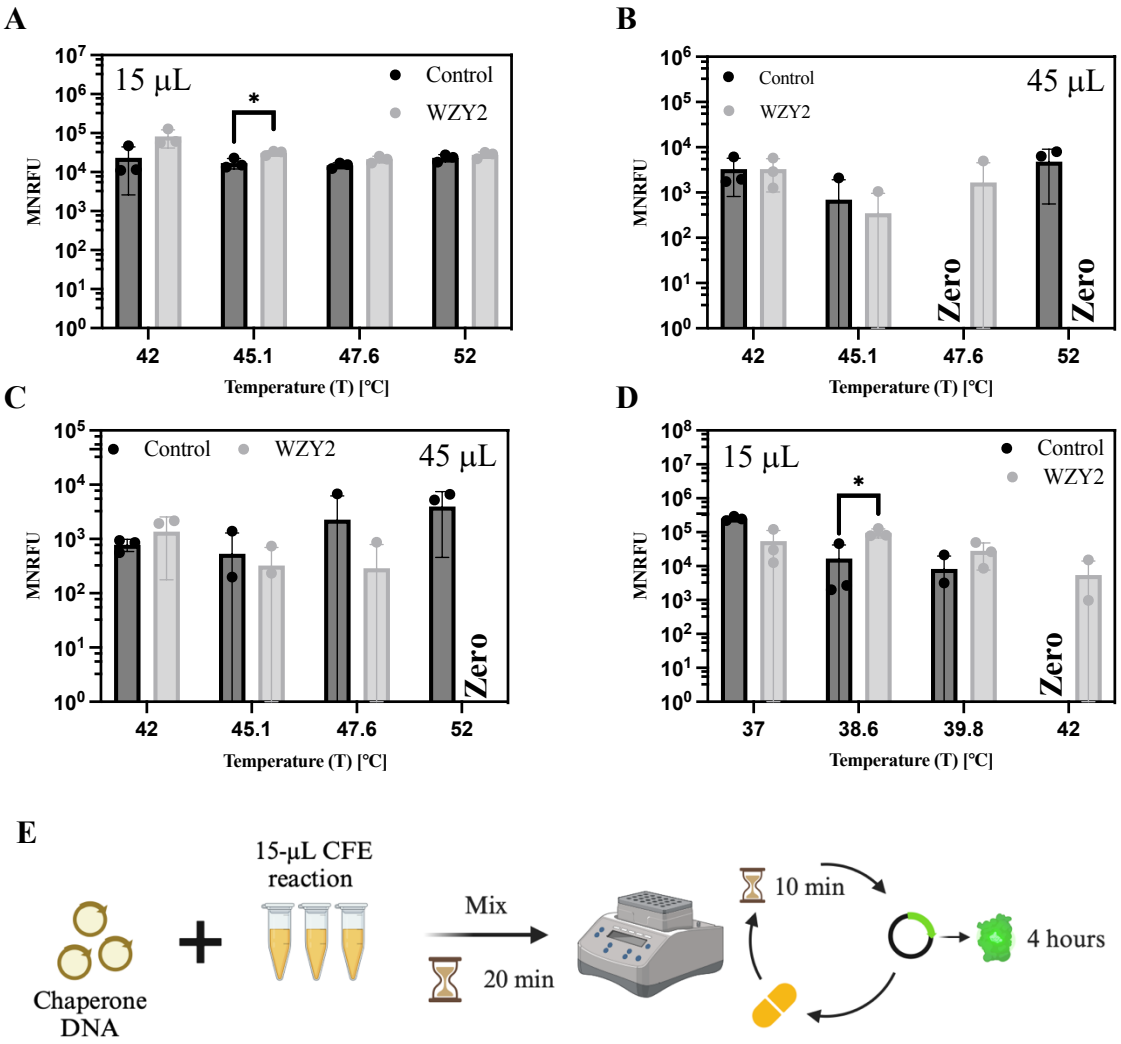


Figure 4.32: Overview of experiments used to generate the novel EGFP expression assay. Temperature and CFE reaction volume were changed while keeping chaperone plasmid DNA concentration the same at 7.5 ng/µL. (A) WZY2, a dehydrin, was assessed in 15µL CFE reactions over the temperature range 42°C to 52°C. (B & C) WZY2 was evaluated in 45 µL CFE reactions over the temperature range 42°C to 52°C. (D) WZY2 was accessed in 15µL CFE reactions over the temperature range 37°C to 42°C. (E) Schematic detailing the EGFP expression assay setup and process. Reported values represent mean normalized fluorescence from 3 replicates (given by the number of dots), and error bars represent standard deviation. Reported statistical significance was found using a standard, unpaired Student's t-test, correcting for unequal variances. '*' indicates a p-value less than 0.05. Panel E was created with Biorender.com.

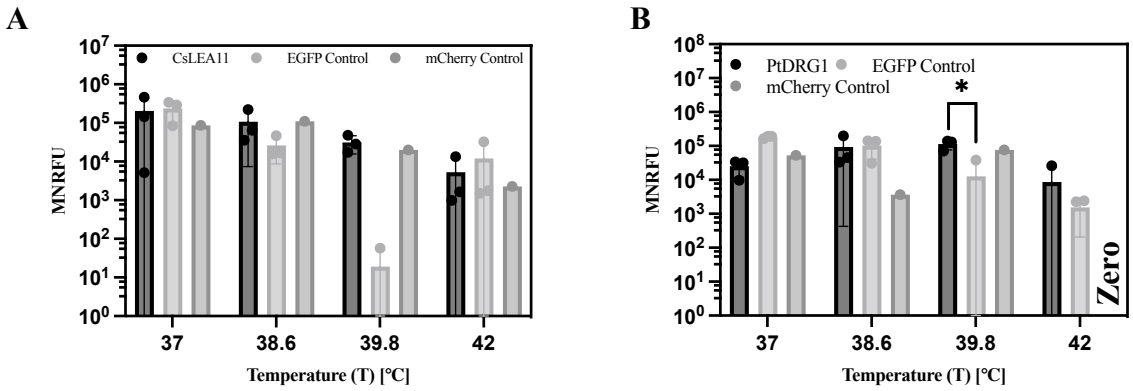


Figure 4.33: Chaperone effects on CFS performance over 37°C to 42°C were evaluated using a novel EGFP expression assay. (A) CsLEA11, a dehydrin, was evaluated in 15µL CFE reactions at a concentration of 5.0 ng/µL. (B) PtDRG1, a desiccation response gene, was evaluated in 15µL CFE reactions at a concentration of 7.5 ng/µL. Reported values represent mean normalized fluorescence from 3 replicates (given by the number of dots), and error bars represent standard deviation. Reported statistical significance was found using a standard, unpaired Student's t-test, correcting for unequal variances. '*' indicates a p-value less than 0.05.

4.4 Discussion

Here, a method was described, namely the novel EGFP expression assay, that successfully integrated chaperone components into CFSs. This assay allowed for the quick screening and study of chaperone components relative to reporter fluorescence output over a wide range of temperatures traditionally thought to impede CFS function and overall system utility. The method described all but eliminates the need for protein purification by supplementing CFSs with chaperone plasmid DNA, not purified chaperone protein, thereby reducing cost- and time-associated burdens in setting up and deploying this system in the field. Admittedly, some EGFP product is lost as the CFS reaction resources are now split between two protein products, the chaperone and the intended protein product. The methods described in Chapter 5 of this thesis address this concern by integrating chaperone protein products into the extract, with some success.

Nevertheless, the methods described and the results identified in this chapter demonstrate the successful integration of four chaperones – Cpn 60/10, WZY2, CsLEA11, and PtDRG1 – into CFSs, with higher reporter fluorescence observed at both low and high temperatures. The results also show that chaperone plasmid DNA concentration can have an inhibitory effect on CFS performance, as was the case for the chaperone components WZY2 and PtDRG1 at 20 ng/μL. Taken together, the results presented in this chapter show that chaperone components can be used successfully in CFSs and might provide a pathway in the realization of fieldable, cell-free protein expression systems due, in part, to the improvement and expansion of the CFS's operating range.

CHAPTER FIVE

TRANSFORMED *E. COLI* VIABILITY ASSESSMENT, CHAPERONE-EXTRACT EVALUATION UNDER TEMPERATURE STRESS, AND CHAPERONE-PRESERVATION ANALYSIS

5.1 Introduction and statement of purpose

This technological approach of incorporating chaperone components into CFSs would be more easily adopted if the system setup and workflow were made more accessible. Therefore, chaperone components had to be incorporated into the cell extract to make this approach more amenable to the target application of these systems – fieldable protein expression. To that end, this thesis chapter's goals were three-fold and meant to illustrate the potential of chaperone functionality when integrated into the cell extract.

First, chaperone components were evaluated in live *E. coli* cells at extreme temperatures to gauge whether they would be helpful to CFS workflows with extracts containing these chaperone components. Second, the efficacy of the transformed extract in CFSs was evaluated by reacting these systems at reaction temperatures as high as 42°C. Third, a preservation assay was carried out with these chaperones by exposing CFS components like the extract and reaction buffer to air-drying and preservation at 37°C for 4 weeks. This timeline was compatible with other published works and satisfied fieldable protein expression requirements. Air-drying methods, not freeze-drying methods, were used as lyophilization machines are prohibitively expensive and not easily operated if deployed in the field.

5.2 *E. coli* viability assessment

5.2.1 Methods & Materials

5.2.1.1 Transformation

The same transformation procedure outlined in A-4 was used here. However, BL21 Star (DE3) cells (Invitrogen, Waltham, MA), not DH5α cells, were used. Glycerol stocks were made with two chaperones, WZY2 and CsLEA11, to verify chaperone functionality in live *E. coli* cells.

5.2.2.2 *Escherichia coli* viability assay

Starter cultures of transformed and non-transformed BL21 Star (DE3) cells were grown overnight with shaking (225 rpm) at 37°C in a 500 mL Erlenmeyer flask containing 100 mL of 2xYPTG media. After that, 2.5 mL of the starter culture was added to a 1 L Erlenmeyer flask containing 250 mL of 2xYPTG media and was incubated until the cultures log phase was reached in a 37°C shaking incubator with a speed of 225 rpm. The cultures were induced with 0.1mM IPTG and were left to grow until three hours of total culture time was met. Then, cultures were diluted to an OD 600 of 1, and 100 μL of cell suspension was added into PCR tubes and were incubated for 20 minutes at the three experimental temperatures - -10, 37 and 50°C. In a 96-well plate, cell suspensions were diluted 10^7-fold and spotted onto 2xYeast-Tryptone (2xYT) plates with and without ampicillin (100 μg/mL) to select for those colonies containing either WZY2 or CsLEA11. Colony-forming units were tallied the next day, following incubation at 37°C for 24 hours.

5.2.2 Results

The translational activity of the lysate has long been equated to the growth rate of cells during harvest [23]. To elucidate whether chaperone components slow *E. coli* growth and whether they aid *E. coli* viability at non-standard temperatures, an *E. coli* growth and viability assay was performed. Growth analyses showed that transformed *E. coli's* growth significantly slowed compared to non-transformed *E. coli*, as much as 3- and 7-times for WZY2 and CsLEA11, respectively (Figure 4.2.21, panels A and C). This result is likely due to the constitutive expression of the chaperone components from the T7Tet repressible promoter, burdening the cells and slowing growth.

In panels B and D of Figure 4.2.21, WZY2 is shown to improve, albeit insignificantly, *E. coli* viability at 50°C by more than one thousand times in the absence of IPTG and by more than 6 times in the presence of IPTG. At -10°C, WZY2 improved *E. coli* viability, in the absence of IPTG, by ~1.59x. CsLEA11, however, is demonstrated to impact *E. coli* viability at all the temperatures tested negatively. For example, the CsLEA11 transformed sample saw a cell viability decrease by 980 times in the absence of IPTG at 50°C and 13.4 times in the presence of IPTG. Interestingly, the CsLEA11 sample was the only one to freeze when left to sit at -10°C for 20 minutes, indicating a relatively low cell count compared to the non-transformed sample.

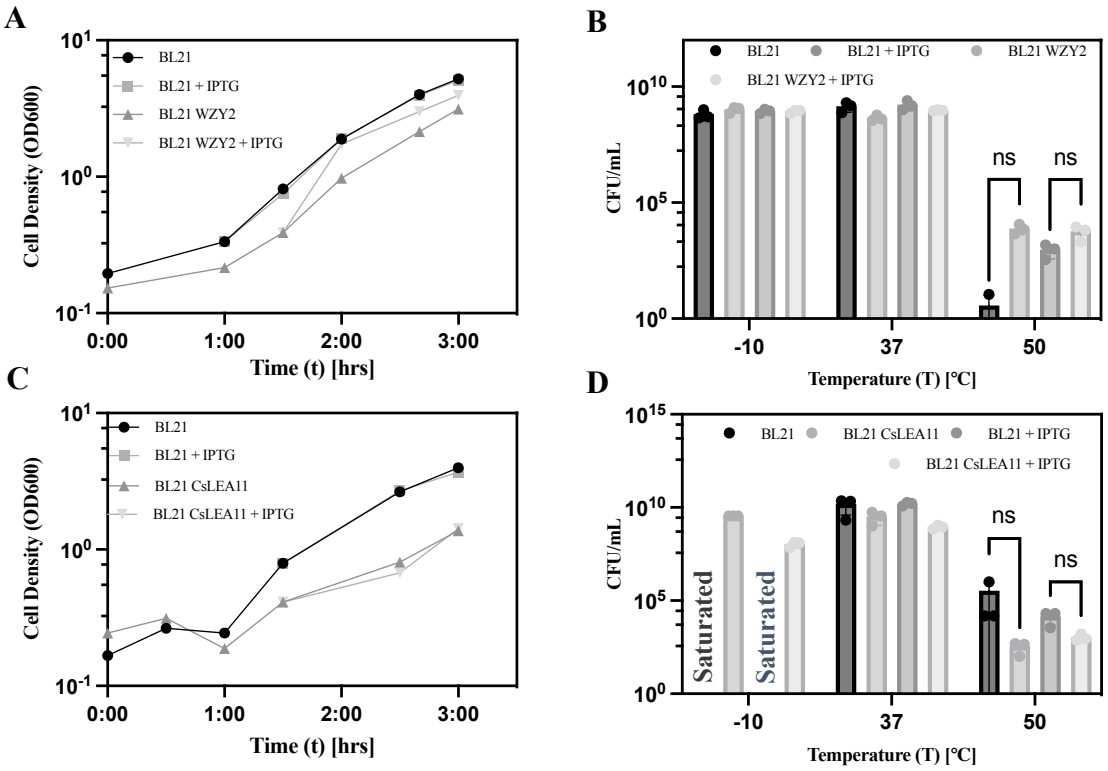


Figure 5.2.21: Effects of chaperone components on *E. coli* growth and cell survival at the temperatures -10, 37 and 50°C. (A and C) Growth curves of transformed and non-transformed BL21 Star (DE3) cells over 3 hours at 37°C with and without IPTG. (B and D) Evaluation of cell survival following sample treatment at the temperatures -10, 37 and 50°C. (B and D) Reported values represent CFU/mL counts from 3 replicates (given by the number of dots), and error bars represent standard deviation. Reported statistical significance was found using a standard, unpaired Student's t-test, correcting for unequal variances. 'ns' indicates no significance.

5.3 Chaperone-extract evaluation under temperature stress

5.3.1 Methods & Materials

5.3.1.1 Extract

Three extract batches, BL21, BL21 WZY2, and E60, were used to generate the results for this thesis section. The methods used to make these extracts can be found in A-2 and those previously published works by Karig and authors [4].

5.3.1.2 Reaction Buffer

The reaction buffer recipe described in A-1 was used here. Magnesium acetate concentrations of 28, 32, and 52 mM were used to generate the results in Figure 5.3.21. Specifically, a magnesium acetate concentration of 32 mM was used to generate panel B, as shown in Figure 5.3.21. A 28 mM magnesium acetate concentration was used for the combined and non-transformed CFS, as supported by panel C in Figure 5.3.21. For the 'completely' transformed CFS, or 'BL21 WZY2', in panels E and F of Figure 5.3.21, a 52 mM magnesium acetate concentration was used.

5.3.1.3 EGFP assay and normalization scheme

The same EGFP assay and normalization scheme described in A-3 of this thesis was used here. However, a unique normalization scheme was performed to generate panel F. Specifically, reported mean fluorescence values for both 'BL21' and 'BL21 WZY2' at each of the temperatures tested were normalized to the highest mean fluorescence values observed in this experiment, which happened to be recorded at 37°C.

5.3.2 Results

Here, the chaperones were expressed in the extract to gauge their efficacy in reacted cell-free systems at elevated temperatures. A growth curve was generated when making the transformed extract. This transformed cell line grew 2 times slower at nearly every growth period documented, an early indication of diminished transformed extract's efficacy in its translational ability downstream when expressing the targeted therapeutic protein or, in this case, a reporter. This reality was observed in panels D, E and F of Figure 5.3.21, wherein the 'BL21 WZY2' CFS underperforms the traditional CFS at every temperature tested. This result aligns with previous chapters, which showed that high chaperone concentrations can significantly impede CFS performance and targeted fluorescence output. It is still unknown whether this result is due to a loss of translational activity of the BL21 WZY2 extract or too much chaperone. It is also demonstrated in panel C that optimal magnesium acetate concentrations differ between extract batch types, especially those containing chaperone components like WZY2. For example, the transformed cell-free system's capacity, or BL21 WZY2, improved 7 times when the magnesium acetate concentration was changed from 8 mM to 26 mM. A ~3x improvement for the CCFS was also observed when the magnesium acetate concentration was changed from 26 mM to 14 mM.

Interestingly, when the chaperone components in the extract are diluted, as seen in panel D of Figure 5.3.21, CFS performance improves compared to the traditional CFS and the completely transformed CFS, or 'BL21 WZY2', at 42°C. This result is encouraging and suggests that the expression of the chaperone component during culture

for extract preparation needs to be attenuated for optimal CFS performance. Moreover, though the completely transformed CFS (CTCFS) performance is worse than the traditional CFS at all temperatures tested, the CTCFS retains its robustness well beyond that of the traditional CFS, as seen in panel F of Figure 5.3.21. For example, at 38.6°C and 39.8°C, the CTCFS maintains 78.4% and 80.7% of its expressive capacity compared to its highest performance measure at 37°C, a result not shared with the CCFS.

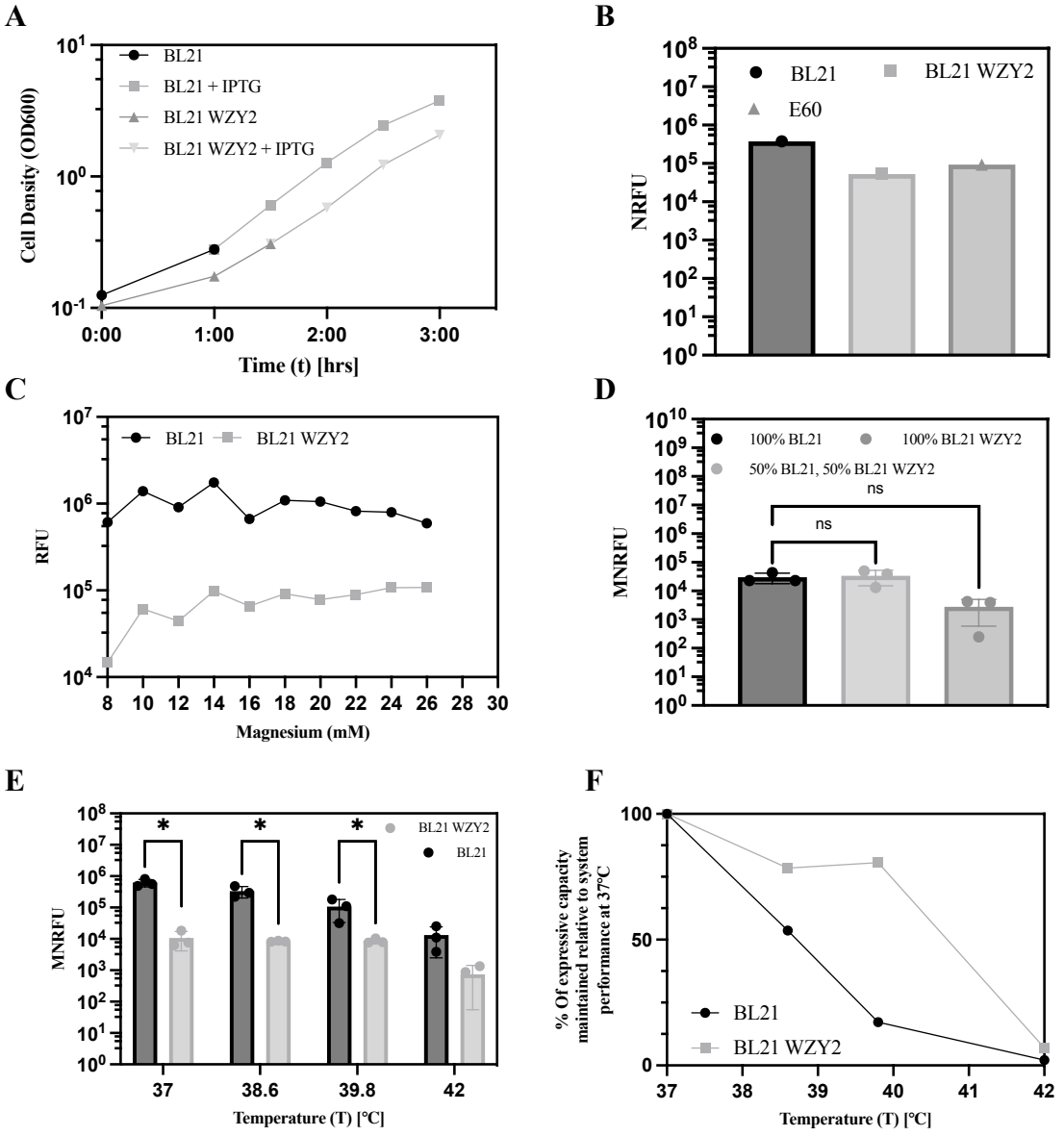


Figure 5.3.21: An assessment of transformed-extract efficacy in supporting CFS performance at elevated temperatures. (A) Growth curves of transformed and non-transformed BL21 Star (DE3) cells over 3 hours at 37°C following induction with 0.1 mM IPTG. (B) A comparison of CFS performance between the extract batches, 'BL21', 'BL21 WZY2', and 'E60' at 37°C. (C) An evaluation of magnesium acetate concentration on CFS performance for two extract batches, 'BL21' and 'BL21 WZY2'. (D) CFS performance according to extract type used, including a mixture of transformed and non-transformed extract, namely '50% BL21, 50% BL21 WZY2', at 42°C. (E) An assessment of WZY2 transformed extract ('BL21 WZY2') on CFS performance at high temperatures. (F) A normalization scheme shows EGFP yield relative to each CFSs

performance at 37°C over the temperature range of 37 to 42°C. In panels D, E and F, reported values represent mean normalized fluorescence from 3 replicates (given by the number of dots), and error bars represent standard deviation. In panels A, B and C, reported values represent singular measurements. Reported statistical significance was found using a standard, unpaired Student's t-test, correcting for unequal variances. '∗' indicates a p-value less than 0.05, and 'ns' indicates no significance.

5.4 Chaperone preservation analysis

5.4.1 Materials & Methods

5.4.1.1 Extract

The extract used in this preservation study was from the batches described in A-2, namely BL21 and BL21 WZY2. For the combined chaperone preserved CFS, or '50% BL21, 50% BL21 WZY2' CFS, the BL21 and BL21 WZY2 extracts were combined in equal amounts to satisfy 15 μL CFE reaction requirements.

5.4.1.2 Reaction Buffer

The same reaction buffer recipe provided in A-1 was used. For the ‘BL21’ and ‘50% BL21, 50% BL21 WZY2’ preserved CFSs, a magnesium acetate concentration of 28 mM was used, as this was found to be the optimal magnesium acetate concentration for these systems, as seen in Figure 5.3.21, panel C. For these same reasons, a magnesium acetate concentration of 52 mM was used in the ‘BL21 WZY2’ preserved CFS.

5.4.1.3 Plate Setup and EGFP expression assay

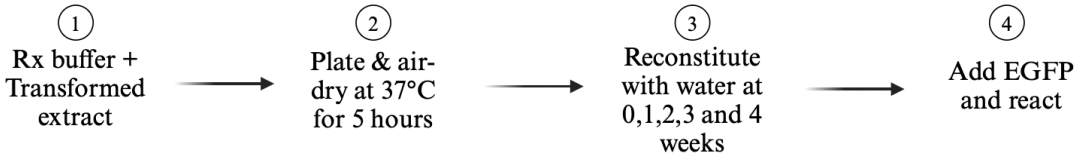
Three aqueous reaction mixtures comprising extract and reaction buffer generated three unique chaperone-preserved CFSs – 'BL21', '50% BL21, 50% BL21 WZY2', and 'BL21 WZY2'. 45 μL of the reaction mixture was plated into 4 wells of a 12-well plate for each of the three systems. Aliquoted reaction mixtures, including those aliquots used for the time zero measurement, were left to air-dry at 37°C for 5 hours. The 12-well plate containing the samples was placed in a 37°C incubator, and the wells were reconstituted with water every week for 4 weeks. Following sample reconstitution with water, the

samples were placed into 12 wells of a Thermo Scientific AB2396 plate, each comprising 11.6 μL of the 15 μL CFS reaction volume. The plate was placed inside the thermocycler, and samples were incubated at 37°C for 10 minutes. 3.4 μL of EGFP DNA was added to the plate, and each well was incubated for 4 hours. Then, kanamycin was added to arrest the reporter's expression. Fluorescence was measured the same way as described in A-3 in addition to how the samples were normalized.

5.4.2 Results

To determine whether chaperones are helpful in preservation efforts, the transformed extract was used to generate two CFSs exposed to air-drying and storage at 37°C for 4 weeks. The chaperone component WZY2 improved CFS performance immediately following reconstitution and at 28 days. At weeks 1, 2, and 3, WZY2 inhibited reporter expression relative to the control - the non-transformed CFS (NTCFS), or 'BL21'. However, during this period, the completely transformed CFS (CTCFS) (‘BL21 WZY2’) retained its robustness, or relative EGFP yield, while the partially transformed CFS (PTCFS) (‘50% BL21, 50% BL21 WZY2) saw a continued and dramatic rise in fluorescence output. At 4 weeks, both the CTCFS and the PTCFS outperformed the NTCFS, and statistical significance was observed between the NTCFS and the PTCFS.

A



B

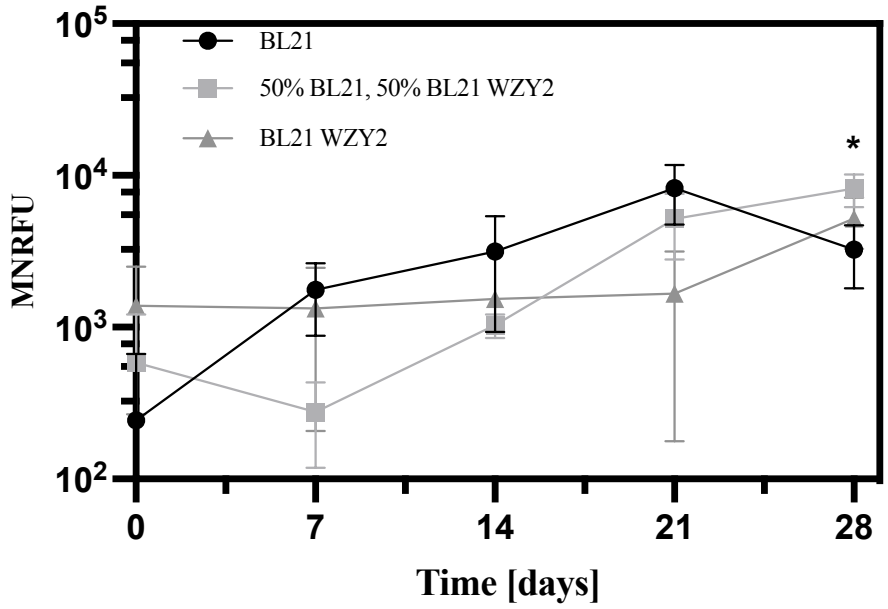


Figure 5.4.21: Analysis of chaperone function in preserved cell-free systems. (A) A step-by-step process detailing the setup of this preservation experiment. (B) Recorded fluorescence for three chaperone-preserved CFSs at 37°C over 4 weeks. Reported values represent mean normalized relative fluorescence from 3 replicates (given by the number of dots), and error bars represent standard deviation. Reported statistical significance was found using a standard, unpaired Student's t-test, correcting for unequal variances. '*' indicates a p-value less than 0.05. Panel A was created with BioRender.com.

5.4 Discussion

Chaperone components, when introduced to *E. coli*, significantly slow growth, primarily due to constitutive chaperone expression from a T7 Tet repressible promoter. WZY2, the wheat dehydrin, not CsLEA11, improves *E. coli* viability at 50°C irrespective of IPTG induction, suggesting that this particular chaperone might be helpful in CFSs as part of the extract at elevated temperatures.

WZY2, expressed in the extract, failed to supply sufficient temperature-stress protection to the CFS at all temperatures tested compared to the conventional CFS. This finding likely resulted from diminished translational activity of the transformed CFS extract, as indicated by slower growth during harvest. However, its robustness, or relative EGFP yield, was maintained up to 39.8°C, a result not seen for the conventional CFS (CCFS). The chaperone WZY2, in this way, positively affects CFS performance at elevated temperatures, though EGFP yield is significantly smaller than observed for the CCFS. It was hypothesized that if WZY2's presence in the extract were lessened, CFS performance would improve. This result was observed at 42°C when WZY2's concentration in solution was effectively halved. It is conclusive that chaperone-transformed extract must be optimized according to CFS reaction conditions and, most likely, can be achieved by repressing, via the Tet operator, chaperone expression during culture in preparation of the extract.

Undoubtedly, the chaperone WZY2 positively affects CFS performance during air-drying and preservation at 37°C. It was observed that the completely and partially transformed CFSs performed better than the CCFS at time zero and at 4 weeks. The

initial data point for the CCFS is thought to be an outlier, resulting from reconstitution discrepancies in a 12-well plate following air-drying. While the exact mechanism of WZY2 function and protection in the completely and partially transformed CFSs is unknown, it is thought that WZY2 protects client proteins, those proteins belonging to the extract, during preservation, such that they are unable to carry forward the translation process of the reporter protein. Only when these suboptimal preservation conditions persist are client proteins able to leach out from the chaperone's protection and act on the DNA template encoding the reporter product. This is believed to be the case as the early performance deficit of the chaperone-preserved CFS cannot be wholly attributed to the loss of translational proteins in the cell extract because they reach equivalent levels of EGFP fluorescence at week 4. This unexpected result suggests that chaperone-preserved CFSs offer a cell-free platform that allows for higher or equivalent EGFP fluorescence later in the preservation cycle, boosting these systems' appeal for preservation times lasting longer than 4 weeks at 37°C. This result warrants further study on the heterologous chaperone function in preserved CFSs.

CHAPTER SIX

CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

This thesis presented novel, functional, and practical methods to ruggedize cell-free protein expression systems and make fieldable protein expression easier. It was demonstrated that heterologous chaperones native to extremophiles can function synergistically with cell-free systems derived from *E. coli* at low and high temperatures and when preserved over long periods. More than this, an experimental scheme named I3 was proposed to allow for the quick study of gene components responsible for chaperone expression, like promoters, ribosomal binding sites, and operator sequences, the chaperone components themselves, and their effects on cell-free system performance in environments of temperature stress or when these systems are preserved via air-drying at 37°C. Together, the results from this work supported the use of chaperone components in CFSs under temperature duress and established an experimental platform that can evaluate chaperone components responsible for more than temperature protection in CFSs, like radiation protection affording chaperones.

6.2 Future Work

Early in this thesis work, it was established that excessive chaperone presence impedes CFS performance, except in CFSs preserved over long periods at 37°C. Future work should take advantage of the TetO sequence introduced to the chaperone plasmid maps at the beginning of this inquiry to curb chaperone expression and concentration found in the extract comprising the CFS to optimize the chaperone-inspired CFS for

improved function at high reaction and preservation temperatures. Additionally, chaperones responsible for functions outside of temperature protection should be tested to gauge whether the I3 scheme is sufficient to elucidate chaperone function in CFSs. Together, this work has the potential to inspire the rapid integration of heterologous chaperone components in CFSs and to make possible a system that can respond to and solve the inherent challenges faced with cell-based modes of protein expression.

APPENDICES

Appendix A

Shared materials and methods

A-1: Media and buffer

1.1 *2xYPTG (Yeast extract, phosphate buffer, tryptone, and glycerol)*

To begin, 16 g tryptone, 10 g yeast extract, and 5 g sodium chloride were added to a 2-L flask with Milli-Q water up to 800 mL. Next, the phosphate buffer was made by combining 2.64 g of monosodium phosphate and 5.68 g of disodium phosphate in a 250 mL flask with Milli-Q water up to 100 mL. These two solutions were autoclaved. The glycerol solution was made by adding 5 g of glycerol to a 250 mL flask with Milli-Q water up to 100 mL. This solution was filtered using a 0.22-micron filter. All three solutions were combined to make 2xYPTG media [4].

1.2 *Buffer A*

Buffer A was prepared by combining the following reagents in a 2-L flask with Milli-Q water up to 1 L: 0.5 mL 2-mercaptoethanol (2-ME), 0.155 g dithiothreitol (DTT), 12.194 g potassium acetate, 3.002 g magnesium acetate, 0.489 g Tris-acetate, and 0.884 g Tris. Buffer A was filtered using a 0.22-micron filter [4].

1.3 *Buffer B*

Buffer B was prepared by combining the following reagents in a 2-L flask with Milli-Q water up to 1 L: 0.155g dithiothreitol (DTT), 12.194g potassium acetate, 3.002g magnesium acetate, 0.489g Tris-acetate, and 0.884g Tris. Buffer B was filtered using a 0.22-micron filter [4].

1.4 *Reaction Buffer*

The reaction buffer used for these CFE reactions contains: 1.2 mM ATP, 0.85 mM of GTP, CTP and UTP, 2 mM DTT, 0.64 mM cAMP, 28.5 mM HEPES-KOH, 90 mM potassium glutamate, 80 mM ammonium acetate, 32 mM magnesium acetate, 34 ug/mL folinic acid, 4 mM of cysteine, 2.1 mM of every other 19 amino acids, 2% PEG (8000), 67 mM creatine phosphate, and 3.2 ug/mL creatine kinase. This reaction buffer recipe is mainly based on the methods proposed by Karig et al., with only minor changes to the magnesium acetate concentration and tRNAs [4].

A-2: Extract

1.4 *BL21 and BL21 WZY2 Extract*

The extract protocol outlined here closely resembles the methods proposed by Karig and colleagues [4]. Briefly, BL21 Star (DE3) (Invitrogen, Waltham, MA) cell starter cultures, with and without the chaperone component WZY2, were grown overnight in 500-mL Erlenmeyer flasks containing 100 mL of 2xYPTG media in a shaking incubator at 225 rpm and 37°C. For the BL21 Star cells transformed with WZY2, ampicillin was added to the culture at 100 μg/mL. The pre-culture (2.5 mL) was then added to 250 mL of 2xYPTG media in a 1-L Erlenmeyer flask. Cultures were left to grow until the log phase was reached, then induced with 1 mM isopropyl β-D-1 thiogalactopyranoside (IPTG). Cells were harvested mid-log phase (OD600 ≈ 4 for the non-transformed culture and ≈ 2 for the transformed culture) and immediately chilled on ice. The chilled cultures were placed in 250 mL Nalgene centrifuge bottles and

centrifuged at 4000xg for 20 minutes at 4°C. Following centrifugation, the supernatant was decanted, and the cell pellets were washed with 20 mL of Buffer A for each gram of wet cell pellet and then repeated twice. Cell pellets were stored overnight at -80°C. The next day, cell pellets were thawed in a water bath at room temperature. Cell pellets were resuspended in 1.27 mL of Buffer B for every gram of cell mass. 1.5 mL of this resuspension was then aliquoted into 2 mL microcentrifuge tubes and sonicated according to these settings: frequency = 20 kHz, amplitude = 50%, pulse on/off cycle = 30 seconds, and total energy per sample = 625 joules. The sonicated cell pellet was centrifuged at 12,000xg for 10 minutes at 4°C. The supernatants were transferred in 1.5 mL to RNase-free 2 mL microcentrifuge tubes and stored at -80°C.

The work of Kwon and colleagues inspired the lysis and post-lysis steps indicated in this protocol. For example, the run-off reaction, following the last centrifugation step, was omitted, as Kwon and colleagues showed that lysate derived from BL21 Star (DE3), the strain used in these extract studies, lost degrees of robustness for run-off reaction times as short as 20 minutes [23]. Additionally, Kwon et al. show that lysate is most productive if, during lysis and sonication, cells receive between 556 and 1102 J of accumulated energy [23].

A-3: EGFP expression assay

Aqueous phase reagents, including extract and reaction buffer, were used for these CFE reactions. The reaction mix was made by combining extract (4.1 μL) and reaction buffer (7.5 μL). This mixture was placed into wells of a Thermo Scientific AB2396 plate. The plate was placed inside the thermocycler for 10 minutes to allow each

well to reach the desired experimental temperature. 3.4 μL of reporter DNA, at a concentration of 44.5 ng/μL, was added to each of the wells with a multichannel pipettor. These triplicate reactions were left to incubate for 4 hours at the desired experimental temperature or temperatures, followed by treatment with kanamycin to arrest reporter expression. The reactions were assayed for fluorescence using a Biotek Cytation 5, with excitation and emission of 479/20 nm and 520/20 nm, respectively. The optics position was set to 'bottom.' All reported fluorescence values have been normalized to background samples containing a reaction mixture of extract, reaction buffer, and water.

A-4: Glycerol stocks, transformation, and DNA purification/extraction

E. coli K-12 glycerol stocks, transformed with the chaperone components, and chaperone plasmid DNA were ordered from Twist Biosciences (CA, USA). The glycerol stocks were ordered so that DNA preparations could be done in-house using the Qiagen Midi prep kit (MD, USA). An in-house glycerol stock was made for the Cpn 60/10 chaperone, as the one received from Twist Biosciences was not viable. Briefly, one vial of DH5α cells was thawed on ice. After thawing, Cpn 60/10 DNA was added to the vial and mixed via tapping. The vial was incubated on ice for 30 minutes, followed by a 30-second incubation period at 42°C. The sample was immediately placed on ice. Super optimal broth (SOC) media (250 μL) was added to the vial and incubated at 37°C for 1 hour at 225 rpm. Following incubation, 20 μL of culture was plated onto Luria-Bertani-ampicillin (100 μg/mL) plates (LB-amp) and incubated overnight at 37°C. Transformants were selected from the plate, added to 5 mL LB with ampicillin (100 μg/mL), and

incubated overnight at 37°C with shaking at 225 rpm. Glycerol stocks were made by adding 250 μL of culture to 250 μL of 34% glycerol. Stocks were stored at -80°C.

Appendix B

Tabulated results from chapter 3

Temperature (℃)	% Of expressive capacity maintained relative to system performance at 37℃
40.2	21%
41.4	5%
42.1	4%
42.5	3%

Table B-1: E60 CFS performance at elevated temperatures relative to its most optimal system performance at 37°C. Cells were sonicated with amplitude set to 60%.

Temperature (℃)	% Of expressive capacity maintained relative to system performance at 37℃
40.2	40%
41.4	28%
42.1	4%
42.5	6%

Table B-2: E80 CFS performance at elevated temperatures relative to its most optimal system performance at 37°C. Cells were sonicated with amplitude set to 80%.

Temperature (℃)	% Of expressive capacity maintained relative to system performance at 37℃
39.4	43%
41.1	16%
42.7	4%
44.3	2%
45.9	1%

Table B-3: BL21 CFS performance at elevated temperatures relative to its most optimal system performance at 37°C. Cells were sonicated with amplitude set to 50%.

Appendix C

Tabulated results from chapter 4

Reaction volume	10 µL (n=3)		15 µL (n=3)		15 µL (n=3)	
Reaction temperature	10°C		45°C		45°C	
Performance improvement compared to the EGFP control	Cpn 60/10	13.7x	CsLEA11 (5 ng/µL)	3.4x	WZY2 (3.33 ng/µL)	1.7x
	mCherry	12.9x			PtDRG1 (10 ng/µL)	2.3x

Table C-1: Chaperone CFS performance relative to the EGFP control at low and high temperatures. Green highlights indicate better system performance for the chaperone system compared to the non-chaperone or conventional system.

Reaction volume	15 µL (n=3)		15 µL (n=3)	
Chaperone (plasmid concentration)	CsLEA11 (5.0 ng/µL)		PtDRG1 (7.5 ng/µL)	
Performance improvement compared to the EGFP control	38.6°C	4.1x	39.8°C	9.0x
	39.8°C	1648x	42°C	5.6x

Table C-2: Chaperone CFS performance relative to the EGFP control at high temperatures. Green highlights indicate better system performance for the chaperone system compared to the non-chaperone or conventional system.

Appendix D

Tabulated results from chapter 5

Comparative growth rates		
BL21 versus BL21 WZY2		
Growth period	– IPTG	+ IPTG
0 to 1 hr	2.2x	-
1 to 1.5 hr	2.8x	-
1.5 to 2 hr	1.8x	1.2x
2 to 2.5 hr	1.8x	1.6x
2.5 to 3 hr	1.2x	1.2x

Table D-1: Growth analysis of BL2l WZY2-transformed cells versus non-transformed BL21 cells at 37℃ with and without IPTG. Red highlights indicate slower growth of transformed cells compared to non-transformed cells during the specified growth period, and green highlights indicate faster growth.

Comparative growth rates		
BL21 versus BL21 CsLEA11		
Growth period	– IPTG	+ IPTG
0 to 0.5 hr	1.4x	-
0.5 to 1 hr	Negative	-
1 to 1.5 hr	2.5x	-
1.5 to 2.5 hr	4.7x	7.1x
2.5 to 3 hr	2.3x	1.3x

Table D-2: Growth analysis of BL2l CsLEA11-transformed cells versus non-transformed BL21 cells at 37℃ with and without IPTG. Red highlights indicate slower growth of transformed cells compared to non-transformed cells during the specified growth period, and green highlights indicate faster growth.

Comparative CFU/mL counts at 50°C					
BL21 WZY2/ BL21	– IPTG	1394x	BL21/ BL21 CsLEA11	– IPTG	980x
	+ IPTG	6.1x		+ IPTG	13.4x

Table D-3: *E. coli* viability analysis of transformed cells (WZY2 and CsLEA11) versus non-transformed cells (BL21). Red highlights indicate diminished cell viability of transformed cells compared to non-transformed cells, and green highlights indicate improved cell viability.

Comparative growth rates		
BL21 versus BL21 WZY2		
Growth period	– IPTG	+ IPTG
0 to 1 hr	2.2x	-
1 to 1.5 hr	-	-
1.5 to 2 hr	-	2.5x

2 to 2.5 hr	-	1.8x
2.5 to 3 hr	-	1.6x

Table D-4: Growth analysis of BL21 WZY2-transformed cells versus non-transformed BL21 cells at 37°C with and without IPTG. Red highlights indicate slower growth of transformed cells compared to non-transformed cells during the specified growth period. These data were used to generate the extract batches, BL21 and BL21 WZY2.

Magnesium effects on CFS performance – a comparison of same-system performance across varying magnesium concentrations		
BL21	26 mM vs. 14 mM	2.94x
BL21 WZY2	8 mM vs. 26 mM	7.3x

Table D-5: Magnesium effects on the BL21 and BL21 WZY2 CFS. Green highlights indicate the degree of system improvement by changing the magnesium concentration.

System performance relative to the control, BL21, at 37°C	
BL21 WZY2	7.2x
E60	4.1x

Table D-6: BL21 WZY2 and E60 CFS performance relative to the BL21 control at 37°C. Red highlights indicate worse system performance for the BL21 WZY2 system and E60 system compared to the BL21 control.

System performance relative to the control, BL21, at 42°C	
50% BL21, 50% BL21 WZY2 (PTCFS)	1.1x
100% BL21 WZY2 (CTCFS)	10.7x

Table D-7: PTCFS and CTCFS performance relative to the BL21 control at 42°C. Red highlights indicate worse system performance for the PTCFS and CTCFS compared to the BL21 control, and green highlights indicate improved system performance.

Temperature (°C)	**% Of expressive capacity maintained relative to system performance at 37°C**	
	BL21	BL21 WZY2
38.6	53.6%	78.4%
39.8	17.2%	80.7%
42.0	2.1%	6.9%

Table D-8: BL21 and BL21 WZY2 system performance relative to their highest system capacities at 37°C.

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