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Cell-free Unnatural Amino Acid Incorporation with Alternative Energy Systems and Linear Expression Templates

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Abstract

Site-specific incorporation of unnatural amino acids (uAAs) during protein synthesis expands the proteomic code through the addition of unique residue chemistry. This field provides a unique tool to improve pharmacokinetics, cancer treatments, vaccine development, proteomics and protein engineering. The limited ability to predict the characteristics of proteins with uAA-incoporation creates a need for a low cost system with the potential for rapid screening. *Escherichia coli*-based cell-free protein synthesis is a compelling platform for uAA incorporation due to the open and accessible nature of the reaction environment. However, typical cell-free systems can be expensive due to the high cost of energizing reagents. By employing alternative energy sources, we reduce the cost of uAA-incorporation in CFPS by 55%. While alternative energy systems reduce cost, the time investment to develop gene libraries can remain cumbersome. Cell-free systems allow the direct use of PCR products known as linear expression templates, thus alleviating tedious plasmid library preparations steps. We report the specific costs of CFPS with uAA incorporation, demonstrate that LETs are suitable expression templates with uAA-incorporation, and consider the substantial reduction in labor intensity using LET-based expression for CFPS uAA incorporation.

Key Words

- Cell-free protein synthesis
- Nonnatural amino acid
- Alternative energy
- Amber codon suppression
- Linear expression templates
- *In vitro* protein synthesis

Abbreviations

- CFPS – cell-free protein synthesis
- uAA – unnatural amino acid
- LET – linear DNA expression template
- pPa – *p*-propargyloxyphenylalanine
- GFP – superfolder green fluorescent protein
- pPaGFP – GFP with T216 to Amber codon mutation

Introduction

The 23 proteomic amino acids have provided for rich biological diversity on earth [1]. Yet the narrow range of chemistries provided by these residues can frequently pose a challenge to the biochemist's quest for site-specific modifications to protein. The site-specific incorporation of unnatural amino acids (uAAs) in proteins unlocks the potential for unique residues. This rapidly growing field provides a unique tool that has already been applied toward improving pharmacokinetics, cancer treatments, vaccine development, proteomics and protein engineering [2-11]. In short, the ability to site-specifically incorporate uAAs is a strong platform to expand the chemistry of life [8, 12-14]. Although decades of work have been devoted to this area of research, major strides have recently been made toward simple, productive, and readily transferable methods of site-specific uAA-incorporation [15-19]. Most notably, Shultz and coworkers have developed a number of evolved aminoacyl tRNA-synthetase/tRNA pairs that act orthogonally to native synthetase/tRNA pairs, allowing for high fidelity protein synthesis without interactions between native and evolved synthetase/tRNA pairs. These evolved synthetases incorporate the uAAs site-specifically at Amber codons [8, 14, 20]. Over 70 uAAs have been incorporated with high specificity using this system [8]. This study employs an evolved synthetase/tRNA pair from *Methanocaldococcus jannaschii* that incorporates the uAA p-propargyloxyphenylalanine [21].

A primary challenge with any uAA-incorporation system is accurately predicting the changes in protein behavior due to the novel residue chemistry provided by an uAA. *In silico* predictions methods are limited and many of the most accurate protein folding predictions algorithms are based heavily on homology, making them less useful when considering novel residue characteristics [22]. Thus the physical screening of many sites may be necessary to find stable, efficacious sites for uAA incorporation and high-throughput screening options would be desirable.

While *in vivo* screening for effective uAA incorporation sites can be successfully employed for high margin applications such as pharmaceuticals, a less expensive screen is desirable for wide spread use of the technology in lower margin applications, such as industrial biocatalysis. In addition, rapid screening would be propitious for applications screening many sites. To address this need, we propose a cell-free approach to site-specific incorporation of uAAs. Cell-free protein synthesis (CFPS) efficiently harnesses the innate ability of the cellular machinery to transcribe and translate while simultaneously allowing superior control over the synthesis environment compared to *in vivo* techniques [23-25]. A cell-free environment provides pronounced advantages for general protein production and uAA-incorporation,

straightforward isotope or other labeling, simplified purification, direct manipulation of reactant concentrations, and high yielding production of toxic proteins or using toxic reactants [26-30].

The attributes of CFPS lend this system to uAA-incorporation screening applications. However, the cost of energizing traditional cell-free systems can be inhibitory for price-sensitive applications such as bulk biocatalyst production, as the energizing reagents often constitute the bulk of the system expense [31]. Traditionally, compounds with high energy phosphate bonds such as phosphoenolpyruvate [32] or creatine phosphate [33] have been used to energize CFPS resulting in a high energy cost per mg protein produced. Alternative energy sources such as glycolytic intermediates have reduced the cost of energizing CFPS and paved a way to make cell-free systems more commercially viable [17, 34]. However, these cost-reduction methods have not yet been reported in connection with uAA-incorporation. Here, we report the impact these energy systems have on uAA-incorporation in CFPS.

While alternative energy systems can reduce the overall cost of CFPS for uAA-incorporation, there remains the time-intensive aspect of producing mutant libraries of plasmid DNA. The use of PCR-synthesized linear DNA is an attractive alternative to using plasmid DNA *in vitro* due to its short preparation time, ease of production, and high-throughput synthesis capacity [35-40]. Using LETs with CFPS eliminates the need for DNA cloning [35], enables rapid high-throughput screening [37-39] and facilitates high-throughput genomic analysis [36, 40]. We report for the first time the use of LETs in connection with batch CFPS for uAA-incorporation and in combination with an alternative energy system. The potential cost- and labor-reductions of uAA-incorporation with CFPS using LETs prepares the way for large scale cost-effective protein screening, conceivably opening the doors to many promising technologies that might be otherwise inhibited by cost.

Materials and Methods

Preparation of cell extract and *E. coli*-orthogonal tRNA synthetase

Cell extract for this work was prepared using *Escherichia coli* strain BL21 Star™ (DE3) cells (Invitrogen, Carlsbad, CA) harboring the pEVOL-*p*PrF plasmid [21]. Cells were grown in shake flask fermentations as previously reported [15]. Fermentations were induced at 0.6 OD_{600} with 1 mM isopropyl β-D-1-thiogalactopyranoside and 0.02% (w/v) L-arabinose to express T7 RNA polymerase and the *E. coli*-orthogonal tRNA synthetase, respectively. Cells were harvested at late exponential phase (OD_{600} 4 to 5.4), lysed with an Emusiflex B-15 French Press (Avestin, ON, Canada) and further prepared as previously described [15].

To further supplement the CFPS reactions, the *E. coli*-orthogonal synthetase was produced *in vivo,* purified, and added as detailed in the supplemental material. The *E. coli*-orthogonal tRNA synthetase was overexpressed from the plasmid pEVOL-*pPr*F harbored in BL21 Star™ (DE3) in shake flasks containing 1 L of 2xYT media at 37 ^{o}C and 280 rpm. Expression was induced with 1 mM IPTG at 0.5 OD_{600} and incubated overnight. Cells were lysed by Emusiflex B-15 French Press (Avestin, ON, Canada) and the synthetase was purified using HisTrap™ HP columns (GE Healthcare, WI).

Preparation of linear expression templates (LETs)

Linear expression templates (LETs) were generated from pY71-sfGFP using two step PCR as previously described [35]. In the first PCR the gene of interest was amplified using gene specific primers. The amplified genes were then advanced to second PCR with ultramers to include the ribosome binding site, T7 promoter and T7 terminator with sequences based on those optimized by Ahn and coworkers [41]. The final PCR product was purified using QIAquick® PCR purification kit following the manufacturer's instruction (Qiagen, Valencia, CA). Plasmid and LETs were used immediately or stored in ddH_2O at -20 ^{o}C until use. The primer sequences used for generating LETs are tabulated in Supplementary Table S1.

CFPS reactions and protein yield determination

Phosphoenolpyruvate (PEP), *E. coli* tRNA mixture, creatine phosphate (CP), and creatine kinase (CK) were purchased from Roche Molecular Biochemicals (Indianapolis, IN) and L-[U-14C] Leucine was purchased from PerkinElmer Inc. (Waltham, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO). *para*-propargyloxyphenylalanine (pPa) was synthesized and characterized as described previously [12]. Energy systems based on the following were used in this work: (1) PANOxSP [42], (2) simplified PANOx (PANOx*) [43], (3) creatine phosphate/kinase (CP/CK) [44], glucose [34], (4) fructose 1,6-bisphosphate (F1,6BP) [17], and (5) glutamate [45]. Detailed initial reaction compositions are described in Supplementary Table S2. Plasmid based reactions were performed with plasmid pY71-sfGFP encoding for a superfolder derivative of green fluorescent protein (GFP) and pY71-sfGFP-T216uAA encoding for pPa incorporation at residue 216 (pPaGFP). The GFP gene was derived from accession number 2B3P_A and the sequence for the expression vector pY71 and the GFP used in this work have been detailed previously [12]. Protein yield was determined using a linearly correlated calibration curve between fluorescence measurement and protein concentration as described previously [15].

Nuclease inhibition

Based on the findings of Amundsen and coworkers, small molecules CID 697851 (IC_{50} of 33 μM, ChemBridge, San Diego) and CID 1517823 (IC_{50} of 5.1 μM, Vitas-M Laboratory, Netherlands) from chemical class cyanothiophene and pyrimidopyridone, respectively, were selected as the experimental nuclease inhibitors [46]. The compounds were first dissolved in DMSO and appropriate dilutions for CFPS reactions were made in DI-water. Reactions were performed using the PANOxSP system augmented with the inhibitors at concentrations specified in Figure 4.

Cost Analysis

The reagent prices used in our calculations were obtained from the 2012 online catalogues of Roche Molecular Biochemical and Sigma-Aldrich. These cost calculations also includes the cost of plasmid or LET DNA preparation for CFPS based on Qiagen Maxiprep kits and PCR reagent costs, respectively. Cost of labor is evaluated based on time-investment only and is not based on monetary units.

Results and Discussion

The expansion of the proteomic code using unnatural amino acids (uAAs) has paved the road for new capabilities in microbiology, proteomics, pharmacokinetics, and biochemical engineering. However, some cost-sensitive applications may be limited by reagent and labor expenses involved with screening and production of uAA-containing proteins. CFPS offers compelling basis for uAA-incorporation due to the open and accessible nature of the reactions. In this work, we capitalize on the open environment *in vitro* systems to incorporate uAA at reduced cost using alternative energy sources. To further the suitability of CFPS for uAA-incorporation, we also report the use of LETs for production of proteins with site-specifically inserted uAA and consider the overall reagent and labor investment. As model proteins, we employed a superfolder green fluorescent protein (GFP) and a derivative of that protein containing a mutation at residue 216 to encode for uAA incorporation (pPaGFP) [12].

Alternative energy sources for Cell-free uAA-Incorporation

The accessible nature of cell-free reactions allows for flexibility of energy sources. Many high yielding *in vitro* systems are expensive due to the price of high energy chemicals such as phosphoenolpyruvate. Indeed, Kim and coworkers reported that in phosphoenolpyruvate-energized CFPS, the energy source accounts for approximately 50% of the total cost [17]. Alternative energy systems based on inexpensive energy-rich molecules, such as other metabolic intermediates, simple carbohydrates and complex

carbohydrates, have been assessed in standard CFPS and have shown promise in reducing the energy costs [16, 17, 34, 47, 48].

Using a batch CFPS uAA-incorporation system, we compared the traditional ATP regeneration system PANOxSP [49] with alternative energy sources based on previous reports such as glucose [34], simplified PANOx (PANOx*) [43], creatine phosphate [50], high glutamate salt system [45], and fructose 1,6-bisphosphate [17] without the use of expensive cofactors such nucleoside triphosphates, acetyl-CoA, and synthetic tRNA . Detailed CFPS reagent compositions are recorded in the supplemental information (Table S2). A single extract was used to provide for consistent comparison across energy systems. In our hands, the overall yields using alternative energy sources were less than 35% of the PANOxSP yield, as seen in Figure 1. This significant reduction in total production offset the savings garnered by the alternative systems in all cases except for the glucose system (Figure 1). Despite a reduction in yield , the glucose system has the lowest reagent cost per yield at $0.658 per 100 μg pPaGFP, owing to the fact that glucose is significantly less inexpensive than PEP and glucose-based yields remained relatively high at 33% of PANOxSP yields (Figure 3).

Linear Expression Templates for Cell-free uAA-incorporation

LET-based expression of uAA-Proteins

CFPS allows for rapid protein production from PCR-produced linear DNA templates (LETs). This prevents the need of plasmid preparation, thus streamlining the creation and expression of mutant libraries. One frequently reported difficulty with the use of LETs is a resulting drop in yields compared to plasmid-based expression [51, 52]. In addition, uAA incorporation consistently has resulted in a drop of full-length protein yields [5, 12, 14, 53]. We therefore hypothesized that the combining of these two technologies would result in compounded loss in yields.

Using PANOxSP as the energy source, LET-based expression of GFP produced 190 μg per mL of CFPS reaction and LET-based expression of pPaGFP produced 50 μg per mL of CFPS. GFP yields were about 15% of the yield of plasmid-based expression (Figure 2). Similarly, pPaGFP yields with LET expression dropped to about 25% of plasmid-based yields. However, yields with PANOxSP were sufficient to detect and analyze pPaGFP in 15 μL CFPS reactions. Our work to reduce reagent costs, improve production yields and provide a detailed cost assessment of uAA-incorporation at these conditions is discussed below.

Effect of Alternative Energy Source on LET-based uAA-incorporation

Based on our results with alternative energy systems in plasmid-based uAA-incorporation, we pursued the glucose energy systems for LET-based expression. With the glucose energy system, the resulting LET-expression yields for GFP were about 18% of PANOxSP yields. For pPaGFP, the LET-expression yields were less than 10% of their PANOxSP counterparts. Despite these stark reductions in protein production, glucose still remained the more economical choice being about 15% less expensive per mg protein due to the considerable price disparity between glucose and phosphoenolpyruvate. However, the glucose system had low yields per volume cell-free reaction, at just 4 μg per mL CFPS. Depending on the application, protein production at these yields may require a larger CFPS volume or more purification steps. This increase in reaction volume required to produce commensurate amounts of protein translates into increased percentage of the cost derived from cell extract. This increase is exemplified in Figure 3, where the cost of cell-extract accounts for nearly 70% of the total reagent cost, while the cost of glucose is less than $0.001 per 100 μg pPaGFP. Increased efficiency of uAA-incorporation could more thoroughly unlock the door for cost-sensitive technologies. Major strides have already been made by Albayrak and Swartz in this area with plasmid-based uAA-incorporation yields increasing based around more efficient tRNA and synthetase systems [54, 55]. Assuming increased efficiency of uAA-incorporation will translate across energy systems, glucose may become a more realistic alternative for energizing uAA-CFPS going forward.

Effect of Nuclease Inhibitors on LET-based uAA-incorporation

The lower yields in LET-based systems have been largely attributed to oligonucleotide degradation by nucleases present in the cell extract [51, 52, 56]. Our *E. coli*-based CFPS system is enhanced for mRNA stability through the *rne131* mutation that results in truncated and inactive RNAse E [57-60]. In addition, the LETs encode a hairpin loop at the 3' end of the mRNA to improve stability and impede exonuclease initiation [41].

Considering the DNA stability, RecBCD is a major *E. coli* DNAse and helicase that likely leads to degradation of LETs [51]. Amundsen and coworkers recently reported the discovery of small molecules that inhibit RecBCD activity [46] . We selected two of the most inhibitory molecules to assess for the first time their impact in our cell-free system (Figure 4) [46]. The addition of these compounds to LET-based CFPS for uAA incorporation increased yields more than 200% in some cases, as seen in Figure 4.

However, yields remained significantly lower than plasmid-based yields, suggesting that RecBCD inhibition by these small molecules is incomplete or other pathways are restricting LET efficacy.

Analysis of Reagent and Labor Costs for LET-based Cell-free uAA-incorporation

In production systems, there are three main concepts to consider for use and viability: 1) reagent expense, 2) product yield, and 3) labor investment. For uAA-incorporation in CFPS, we have considered reagent expense and found that alternative energy systems can significantly reduce costs while maintaining sufficient yields for general analysis in the case of pPaGFP. Figure 3 illustrates the individual and total reagent costs in USD per 100 μg of proteins produced for PANOxSP and glucose energy systems. While PANOxSP systems work well for LET-based CFPS, the lower yields achieved by glucose systems may complicate purification and analysis. Notably, LET-based CFPS uAA-incorporation is 4-8 times more expensive per μg protein than plasmid-based expression. However, this cost-estimate is based only on reagent prices and does not consider the difference in labor investment.

LET-based CFPS can significantly reduce labor investment of protein production when compared to *in vivo* or plasmid-based CFPS, particularly when creating PCR-based libraries of mutants, as illustrated in Figure 5. For *in vivo* expression systems, a rationally designed mutant gene is synthesized by PCR, and subsequently must be digested, transformed, plated, selected, prepared, and transformed into the expression strain before cell growth and maintenance can begin. For plasmid-based CFPS, the labor intensity is reduced by removing the needs to transform into an expression strain and perform cell maintenance. LET-based CFPS drastically reduces the labor intensity by directly adding the PCR products to the CFPS reaction. In addition, *in* vivo preparations require time-consuming waiting periods during plating, colony selection and initial inoculation. In contrast, CFPS reagents such as extract and energy source can be stockpiled for immediate use. Furthermore, lower uAA-incorporation efficiency (i.e. lower amber codon suppression efficiency) due to low uAA transport or competition with endogenous processes can necessitate larger reaction volumes and/or longer cell maintenance for *in vivo* systems.

Purification and analysis in CFPS systems also benefit from the pre-reaction removal of unnecessary cellular debris such as the cell wall, genomic DNA, and unwanted mRNA. This reduces labor intensity by removing steps such as cell lysis and centrifugation while reducing obfuscating background expression. The disparity in labor intensity between *in vivo* preparations and LET-based CFPS for uAA would likely be exacerbated by efforts to screen large libraries.

Based on our experience, LET-based CFPS allows for gene mutants to be produced as proteins within 6-12 hours of commencing PCR. This accelerated time to production allows for creation of gene mutants, production of protein, and analysis of protein in a single day. In contrast, our experience is that plasmid-based mutants in CFPS require a minimum of 2 days to produce and *in vivo* expression typically requires more than 2 days. In short, LET-based CFPS has the potential to significantly reduced time to analysis of proteins.

The time and price economics reported here provide a snapshot of expected expenditures using a commercially available *E. coli* strain and facile fermentations conditions for extract preparation. In this rapidly expanding field of study, the uAA-incorporation yields continue to increase through technological advancements. Recently, Albayrak and Swartz were able to considerably increase plasmid-based uAA-incorporation yields by coexpressing components required for uAA insertion during CFPS [55]. Continued improvements such as reported by Albayrak and Swartz could be adapted to LET-based CFPS to expand the practicable applications of cell-free uAA-incorporation to lower margin applications.

Overall, cell-free uAA-incorporation is a versatile platform that allows for low-cost protein production with alternative energy systems and rapid gene deployment through the use of LETs. The technology described herein opens new avenues for rapid screening and has the potential for automation and high-throughput applications. The developments reported here could aid in lowering costs and expediting uAA applications in biocatalysis, pharmaceutical, and medical diagnostic applications where preliminary work with uAAs has enabled marked improvements.

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Supplemental Information

Supplemental information contains descriptions of primers employed in this work (Table S1) and specifications of energy system components (Table S2).

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Figures

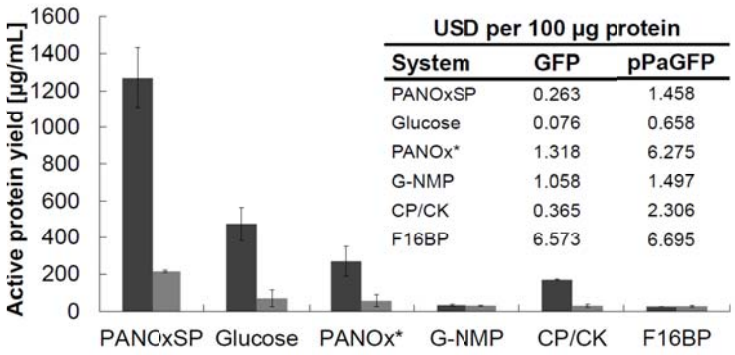

Figure 1: Active GFP and pPaGFP Yields produced with Plasmids and PANOxSP or Alternative Energy Systems.

GFP (dark grey) and pPaGFP (light grey) were produced in CFPS from plasmids and yields were assessed by fluorescence activity. The cost per yield is based on yields and reagent prices. Details of the reaction components and concentrations can be found in the supplementary information. n=3, error bars represent standard deviation.

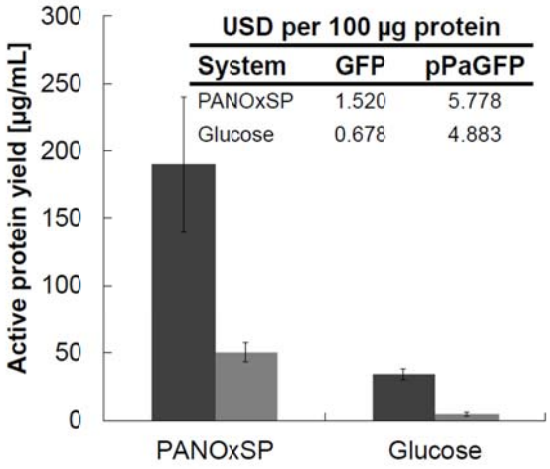

Figure 2: Active GFP and pPaGFP Yields produced with LETs and PANOxSP or Glucose Energy Systems. GFP (dark grey) and pPaGFP (light grey) were produced in CFPS from LETs and yields were assessed by fluorescence activity. Details of the reaction components and concentrations can be found in the supplementary information. n=3, error bars represent standard deviation.

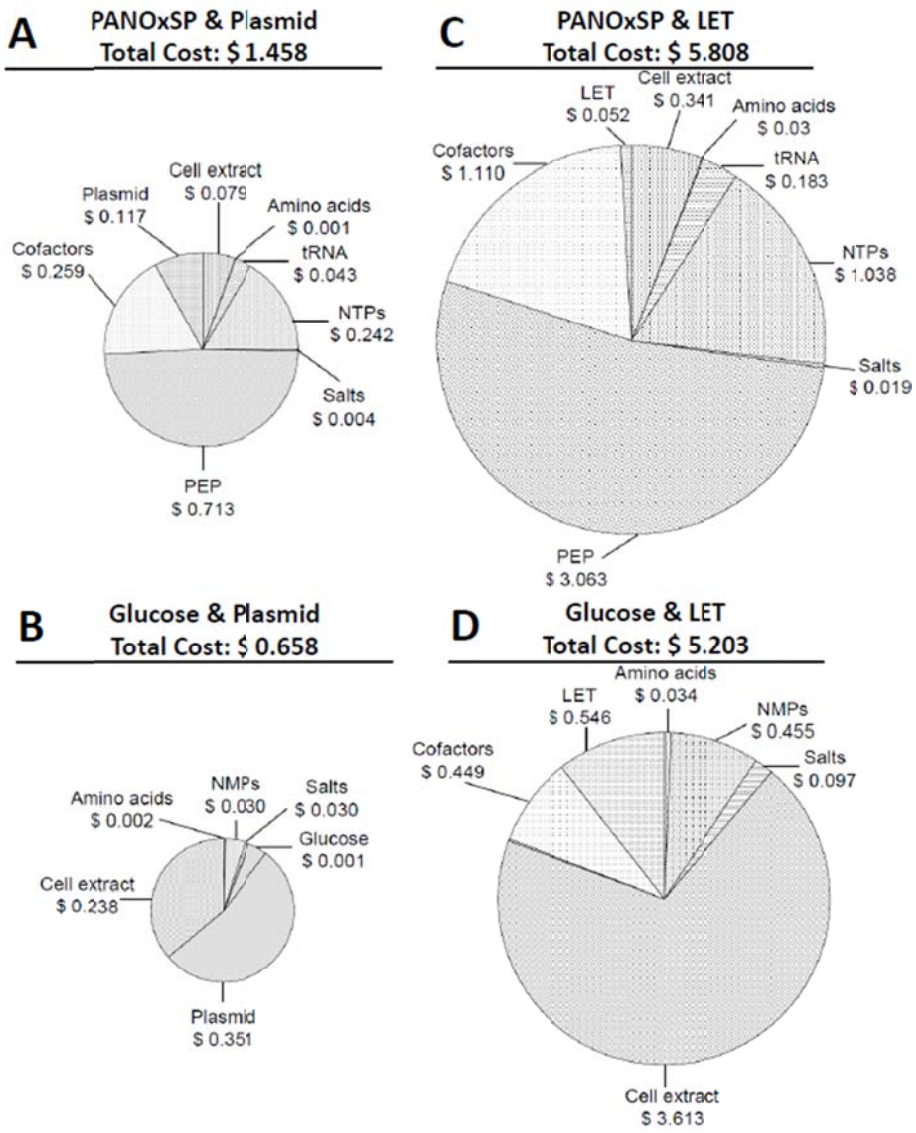

Figure 3: Reagent Cost Analysis for PANOxSP and Glucose Energized CFPS to Produce 100 µg of pPaGFP.

The total reagent cost per 100 µg pPaGFP (listed above each graph, respectively) was broken down into individual component costs of the energizing systems. Details regarding the reaction components, concentrations, and assumptions in calculating the reagent costs are detailed in the materials and methods section and supplementary information.

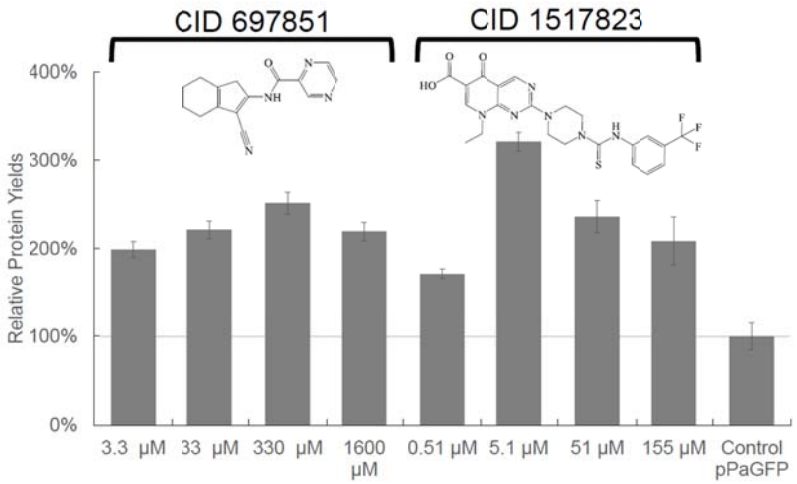

Figure 4: LET-CFPS Relative Yields of Active pPaGFP in the Presence of RecBCD Inhibitors. The chemical structures of the respective inhibitors are depicted. The inhibitor concentration in the cell-free reaction is listed on the x-axis. The control reaction was identical save the replacement of the RecBCD inhibitor solution with water. Error bars represent the standard deviation of three experiments.

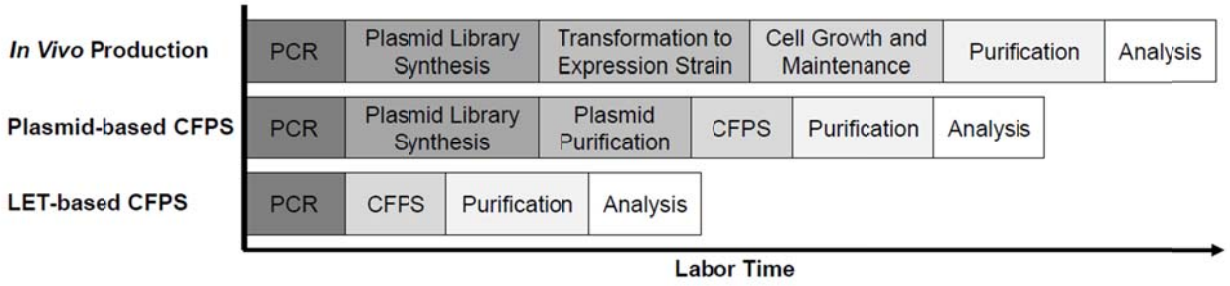

Figure 5: Comparison of Labor Investment for *in vivo* Expression, Plasmid-based CFPS, and LET-based CFPS. CFPS decreases labor by reducing transformation steps and simplifying purification and analysis. LET-based CFPS completely removes the need to rely on live cells during the protein production process. Cell-extracts can be stockpiled and rapidly deployed for synthesis reactions.